Volume 45 *June 1959* *Number 6*

Proceedings of the
National Academy of Sciences
of the United States of America

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THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
is published monthly by
THE NATIONAL ACADEMY OF SCIENCES

Proceedings of the

NATIONAL ACADEMY OF SCIENCES

Volume 45 · Number 6 · June 15, 1959

HYDROMAGNETIC EQUILIBRIUM II. STABILITY IN THE VARIATIONAL FORMULATION

By L. Woltjer

THE INSTITUTE FOR ADVANCED STUDY

Communicated by S. Chandrasekhar, April 3, 1959

In a few recent papers (Woltjer,[1, 2] Chandrasekhar[3]) a beginning has been made with a variational formulation of the problems of hydromagnetic equilibrium. A similar approach was followed by Kruskal and Kulsrud[4] who examined the static equilibrium in a toroid from the variational point of view, making largely use of topological arguments. It seems somewhat doubtful whether this approach can be generalized to nonstatic equilibria in a more general geometry.

The basic idea behind the variational formulation is the following. The hydromagnetic equations (in the absence or dissipative terms) admit certain integrals of motion. A hydromagnetic system cannot change into an arbitrary different system, but only into systems characterized by the same integrals. Thus the integrals may be considered to be the constraints of the system. If now the energy has a minimum value compatible with the constraints, the system must be in stable equilibrium. For a change of configuration would mean an increase in energy, which is not possible since the energy is conserved. An alternative formulation of the variational principle is that if a system is able to dissipate energy, without changing the other integrals of motion, it will move toward a state of minimum energy, compatible with the constraints.

The advantages of a variational formulation are twofold. The relation between the various possible equilibria becomes more clear. Also a variational principle is useful in computing configurations that cannot be dealt with analytically.

Two points in our previous papers need some clarification. Firstly, it is clear that our set of integrals is not complete. As shown by Kruskal and Kulsrud, the variational principle should give all possible equilibrium solutions, if a complete set of integrals is used as constraints. The fact that we did not obtain all solutions demonstrates that our set of integrals is not complete.* Although the relevance of various integrals may depend on special symmetries in the geometry of the problem, there is no guarantee that no more important solutions exist. This remark applies equally well to our discussion[2] as to the one by Chandrasekhar[3] who discussed axisymmetric incompressible media. In fact for this case a complete set of integrals can be obtained[5] and it can be shown explicitly that the variational formulation and the direct solution of the equilibrium equations lead to identical results.

The second point which needs some amplification and which was stated incor-

rectly in our previous papers concerns the stability of the equilibria. Obviously minimizing the energy, we obtain stable configurations. But the variational principle gives us true minima as well as other extrema, the latter corresponding to unstable equilibria. Thus stability occurs only for certain values of the Lagrangian multipliers in our equations. Generally the nature of an extremum and thus its stability can only be established by determining the second variation of the energy. However, in some cases, we can find the most stable configuration, i.e., the state for which the energy is an absolute minimum, by simpler means. Let us consider the force-free magnetic fields with constant α, given by the equation

$$\text{curl } \mathbf{H} = \alpha \mathbf{H}. \tag{1}$$

They are obtained[1] on minimizing the magnetic energy

$$\mathfrak{M} = \frac{1}{8\pi} \int |\text{curl } \mathbf{A}|^2 \, d\tau \tag{2}$$

with respect to the integral of motion

$$I_1 = \int \mathbf{A} \cdot \text{curl } \mathbf{A} \, d\tau, \tag{3}$$

where A represents the vector potential. From these equations we find

$$\mathfrak{M} = \alpha / 8 \pi I_1 \tag{4}$$

Now the explicit solution of equation (1) with the boundary condition $\mathbf{H} \cdot \mathbf{n} = 0$ shows that α must be a zero of some Bessel function $j_n (n \geq 1)$. An infinite number of such α's exists but the α which corresponds to the first zero of j_1 is the smallest one. Therefore for this α the energy (4) attains its minimum value. Thus the lowest mode of the force-free field corresponds to a true minimum and is therefore stable. Explicitly this has been shown to be the case if the disturbances are axisymmetric.

Next we consider the incompressible medium. We shall neglect first the angular momentum integrals. It was shown in our previous paper[2] that in this case a second relevant integral is

$$I_2 = \int \mathbf{H} \cdot \mathbf{v} \, d\tau \tag{5}$$

where $\mathbf{v}$ is the velocity in the medium. On minimizing the energy

$$E = \int \left\{ \frac{1}{2} \rho v^2 + \frac{H^2}{8\pi} \right\} d\tau \tag{6}$$

with respect to the integrals I_1 and I_2 the equations for the magnetic- and velocity-fields become

$$\text{curl } \mathbf{H} = \frac{\alpha}{1 - 4 \pi \beta^2 / \rho} \mathbf{H} \tag{7}$$

$$\text{and } \mathbf{v} = \frac{\beta}{\rho} \mathbf{H}. \tag{8}$$

In the singular case $4 \pi \beta^2 = \rho$, $\alpha = 0$ we directly obtain

$$E = \beta I_2 = \left(\frac{\rho}{4 \pi} \right)^{1/2} I_2 \tag{9}$$

If $\alpha \neq 0$ the magnetic field again must be force-free. From equations (7) and (8) we then have

$$\frac{\alpha}{1 - 4\pi\beta^2/\rho} I_1 = \frac{\rho}{\beta} I_2 \tag{10}$$

The total energy expressed in the integrals of motion becomes

$$E = \left(\frac{1}{2}\beta + \frac{\rho}{8\pi\beta}\right) I_2 \tag{11}$$

Thus the energy is a minimum if $\rho = 4\pi\beta^2$. However, since now $\alpha/(1 - 4\pi\beta^2/\rho)$ has to be a zero of a spherical Bessel function, the possible β's form a discrete set and to have minimum energy we must choose the β closest to the β which satisfies $\rho = 4\pi\beta^2$. Thus the energy in this case will usually exceed the energy corresponding to $\rho = 4\pi\beta^2$ which again is βI_2. Thus the singular solution $\alpha = 0, 4\pi\beta^2 = \rho$ is the solution of lowest energy and should be stable. In fact, Chandrasekhar[6] has shown explicitly that this is the case. Now in this equipartition solution the field is still unspecified, apart from the condition that it must give the correct integrals. It should be noted that this solution is only possible if

$$\frac{\rho}{\beta} I_2 > \alpha_0 I_1 \tag{12}$$

since the magnetic energy cannot be smaller than $\alpha_0/8\pi I_1$, where α_0 corresponds to the lowest force-free mode.

If more integrals are of interest the minimum energy will generally increase. However, the freedom in the choice of the magnetic field makes it clear that many integrals can be satisfied in the equipartition solution. Thus frequently this will be the state of lowest energy available to a system. This may give some indication of a tendency toward equipartition of energy between the magnetic field and the velocity field in an equilibrium configuration, something which does seem not improbable on general grounds. The same tendency toward equipartition is also exhibited by the force-free solutions with motions. The energy (11) is a minimum if β is close to $(4\pi/\rho)^{1/2}$, which means from equation (10) that the energy is lower in the higher modes of the force-free field than in the lower ones if I_2/I_1 is large. And in these modes we again approach equipartition.

The author's stay at the Institute for Advanced Study was supported by the Netherlands Organization for Pure Research (Z. W. O.). Additional support was received from the Institute for Advanced Study.

* I am indebted to Dr. R. M. Kulsrud for valuable criticism on this point.

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*ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. VI. INFLUENCE OF BACTERIOPHAGE T2 ON THE SYNTHETIC PATHWAY IN HOST CELLS**

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Communicated April 20, 1959

Information now available about DNA[1] synthesis by *Escherichia coli* enzymes[2–4] has encouraged an inquiry into the biochemical basis for the observation that a phage-infected *E. coli* cell ceases to produce its own DNA and makes instead the DNA characteristic of the infecting phage.[5] This general problem poses several rather specific questions which may be summarized as follows:

1. T2, T4, and T6 DNA differ from the DNA of *E. coli*, as well as from that of other sources which have been examined, in containing hydroxymethylcytosine (HMC) but no cytosine.[6] Flaks and Cohen[7] have already shown that within several minutes after infection by phage T2, T4, or T6, a new enzyme which hydroxymethylates deoxycytidine 5′-phosphate is produced. Is there an enzyme for converting the resulting dHMC-5-P to the triphosphate level in order to provide a functional substrate for DNA synthesis?

2. With respect to the exclusion of cytosine from the DNA of phage T2, T4, and T6, is there a mechanism in the infected cell for removal of deoxycytidine triphosphate from the site of polymerase action?

3. The DNA's of T2, T4, and T6 contain glucose linked to the hydroxymethyl groups of the HMC in characteristic ratios,[8, 9, 10] although it is clear that in T2 and T6 some of the HMC groups contain no glucose.[9] According to our present understanding of DNA synthesis,[4] it is difficult to conceive how these constant ratios are achieved if the incorporation were to occur via glucosylated and non-glucosylated HMC nucleotides. Is there an alternative mechanism involving direct glucosylation of the DNA even though direct substitutions on intact DNA have been hitherto unknown?

4. Following phage T2 infection there is a temporary halt followed by a resumption of DNA synthesis at about 5 times the rate shown by the uninfected cell.[11] However, measurements with extracts of infected cells, using standard substrates, revealed much diminished rather than the anticipated augmented levels of DNA-synthesizing activity.[12] What are the altered conditions for assay of DNA synthesis in infected cell extracts which would elicit the high levels of activity expected from the physiologic studies?

We have explored these questions and have found that following infection with phage T2 several new enzymes appear.[13] These are (1) an enzyme which phosphorylates dHMC-5-P, leading to the synthesis of hydroxymethyldeoxycytidine triphosphate (dHMC-TP), (2) an enzyme which removes the terminal pyrophosphate group from dCTP, and (3) an enzyme which transfers glucose from UDPG directly to the HMC in DNA. Measurements of DNA synthesis, using dHMC-TP in place of dCTP, revealed about a 12-fold increase in activity in extracts of infected cells over the levels observed in uninfected cell extracts.

METHODS AND MATERIALS

Preparation of Cell Extracts.—E. coli B was grown at 37° with vigorous aeration in M-9 medium[14] modified to contain per liter: KH_2PO_4, 3 gm, Na_2HPO_4, 6 gm, NH_4Cl, 1 gm, $MgSO_4 \cdot 7H_2O$, 0.49 gm, glucose, 5.0 gm, $FeSO_4 \cdot 7H_2O$, 0.5 mg, $CaCl_2$, 55 mg. Growth at a logarithmic rate continued to 4–5 $\times$ 10^9 cells per ml with a generation time of about 50 min.

Cell extracts were prepared in two ways:

Method I: Cultures grown to 2 $\times$ 10^8 cells per ml were chilled, centrifuged, and the cells resuspended at 4 $\times$ 10^9 cells per ml in cold growth medium. Five T2r$^+$[15] or T5 per cell were added and after a 4-min adsorption period at 0°, the culture was diluted twenty-fold into fresh growth medium at 37° and aeration continued. The time of dilution was taken as "zero minutes." 50-ml aliquots were pipetted rapidly onto crushed ice at intervals. The cells were sedimented by centrifuging for 5 min at 10,000 $\times$ g, were resuspended in 1 ml. of 0.5 *M* glycylglycine buffer, pH 7.0, containing 0.001 *M* glutathione, and were stored for 1 to 3 days at −15°. The cells were disrupted in a 10 kc Raytheon sonic oscillator. After removal of a small amount of debris by centrifugation, the extracts contained about 2 mg of protein per ml.

Method II: Cultures were grown to 2 $\times$ 10^9 cells per ml, in the modified medium without $CaCl_2$ and four T2r$^+$ per cell added ("zero minutes"). 50-ml aliquots were pipetted rapidly onto crushed ice at intervals. The cells were sedimented and resuspended in 4 ml of 0.05 *M* glycylglycine buffer, pH 7.0, containing 0.001 *M* glutathione and disrupted as above. Extracts containing about 6 mg of protein per ml were obtained after centrifugation.

All results refer to extracts prepared by Method I unless otherwise stated. While Method II was less effective for phage multiplication (see below), a description of this method is included since it provided an alternative and efficient technique for obtaining concentrated extracts in kinetic studies. The activities per mg protein for the several enzymes studied were found to be at levels similar to those obtained by Method I.

Bacteriophage Determinations.—Bacteriophage was assayed by standard techniques.[16] Intracellular phage was measured after "lysis from without" essentially as described by Doermann.[17] The formation of infectious units in both T2r$^+$ and T5 infected cells (Fig. 1) was found to proceed in normal fashion in cells infected as described in Method I. T2r$^+$-infections produced by Method II yielded only about 2 phage per original cell at 25 min when measured after "lysis from without," although after clearing of the culture a yield of several hundred phage per original cell was obtained.

Enzyme Assays and Preparations.—Phosphorylation of the deoxynucleoside monophosphates (kinase activities) was measured, as described before,[2] by using a 5′-P^{32}-labeled mononucleotide as substrate and assaying the amount of label which becomes resistant to the action of semen phosphatase.[18] Formation of dHMC-5-P from dC-5-P (hydroxymethylase) was assayed according to Flaks and Cohen.[7] The assay of DNA synthesis ("polymerase") was measured by the conversion of a C^{14}-labeled deoxynucleoside triphosphate into an acid-insoluble product.[2] "Polymerase" fraction VII from uninfected *E. coli* was prepared as previously described.[2]

Substrates.—Deoxynucleotides and samples of native and enzymatically synthesized DNA were prepared as in earlier studies.[2, 4] dHMC-5-P was synthesized according to Flaks and Cohen[7] using a 230-fold purified hydroxymethylase[19]; C^{14}-dHMC-5-P was obtained by using C^{14}-formaldehyde (Volk Radiochemical Co.) in the hydroxymethylation reaction, and P^{32}-dHMC-5-P was prepared by using P^{32}-dC-5-P. The E_m value determined for the nucleotide in this preparation was 13.5×10^3 at 284 mμ at pH 1. Since this value conflicts with that of 11.7×10^3 given by Flaks and Cohen,[7] it is regarded as provisional and requires further

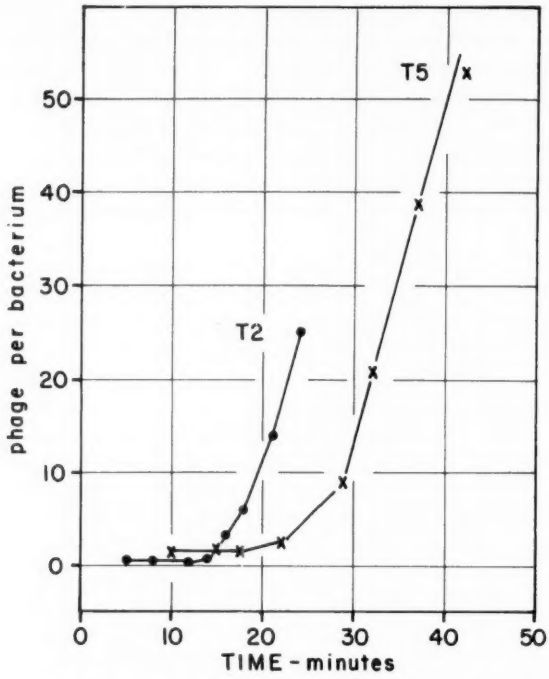

FIG. 1.—Appearance of phage in T2- or T5-infected cells. Infected cultures were prepared by Method I. Infectious units were measured after "lysis from without."

checking. C^{14}-glucose 6-phosphate was prepared by hexokinase action on uniformly labeled C^{14}-glucose (Isotope Specialties Co.). C^{14}-UDPG was prepared from C^{14}-glucose 6-phosphate and uridine triphosphate by the action of phosphoglucomutase and UDPG pyrophosphorylase as outlined by Glaser and Brown.[20] Unlabeled UDPG was a product of the Sigma Chemical Company.

RESULTS

An Enzyme which Phosphorylates dHMC-5-P.—At about 4 min after infection of *E. coli* with phage T2, it was possible to detect in the extracts an enzyme which catalyzes the phosphorylation by ATP of dHMC-5-P (Fig. 2 *A*). This reaction was

undetectable (<0.5 per cent of the maximal value after infection with T2) in normal cells or in extracts of cells infected with T5 (Fig. 2 *A*), a phage without HMC in its DNA. The maximal level of phosphorylating ("kinase") activity for dHMC-5-P was of the same order as that of the kinases for the other deoxynucleotides in-

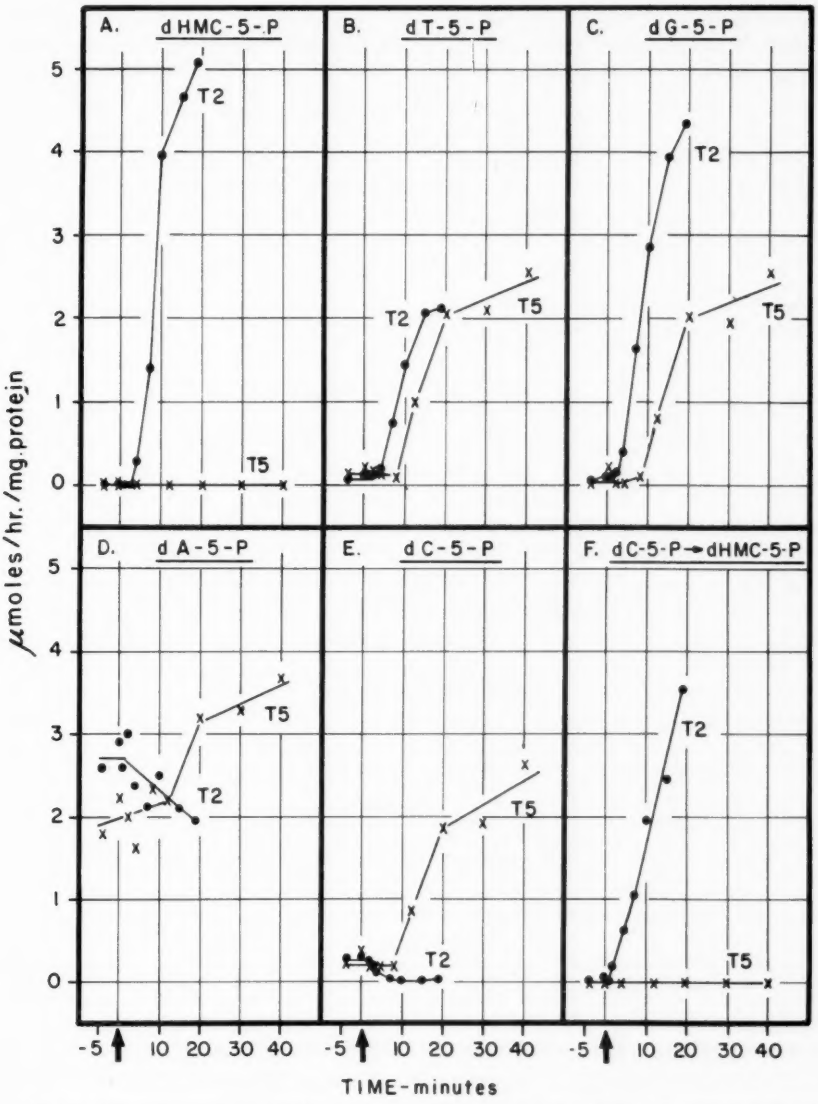

FIG. 2.—Deoxynucleotide-phosphorylating enzymes ("kinases") and hydroxymethylating enzyme levels before and after infection with phage T2 or T5. The arrow indicates the start of infection ("zero minutes," see Method I). Assays were as referred to in Methods.

corporated into viral DNA (see below) and was essentially similar in extracts prepared by either Method I or Method II.

The product of the dHMC-5-P kinase action (with the presumed participation of nucleoside diphosphate kinase in the preparation) was shown to be the triphosphate. Using a 20-fold purified kinase preparation,[19] 25 μmoles of dHMC-TP were prepared and isolated by ion-exchange chromatography, a yield of 92 per cent based on the starting dHMC-5-P. Theoretical specific radioactivity values were found in the isolated dHMC-TP when P^{32}- or C^{14}-labeled dHMC-5-P was the

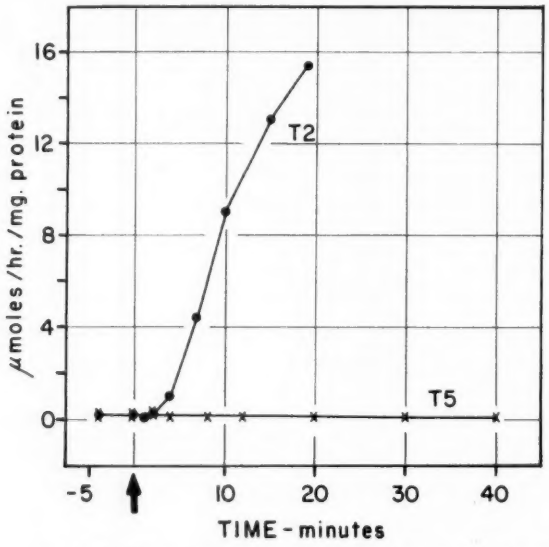

FIG. 3.—dCTPase levels before and after infection with phage T2 or T5. The arrow indicates the start of infection ("zero minutes," see Method I). The incubation mixtures (0.25 ml) contained: 6.0 mμmoles of dCTP labeled with P^{32} in the terminal pyrophosphate group (10^3 cpm per mμmole), glycine buffer, pH 9.2 (0.04 *M*), $MgCl_2$ (0.008 *M*), 2-mercaptoethanol (0.01 *M*) and a quantity of extract containing about 0.3 μg of protein. After 20 min of incubation at 37°, the reaction was terminated by adding 0.5 ml of 0.1 *N* HCl, followed by 0.2 ml of a mixture (containing per ml: 5 mg of crystalline bovine serum albumin, 25 μmoles of sodium pyrophosphate and 25 μmoles of potassium phosphate, pH 7), and 0.1 ml of a Norit suspension (20 per cent packed volume). The Norit was removed by centrifugation and the supernatant fluid was assayed for radioactivity.

starting material. After concentration with Norit, analysis revealed a ratio of HMC: total P: acid-labile P of 1.0: 3.2: 2.2, using an E_m value of 13.5×10^3 at 284 mμ at pH 1.

Kinase Levels for the Other Deoxynucleotides and Levels of the Hydroxymethylating Enzyme.—It is noteworthy that after T2 infection, the kinase levels for dT-5-P and dG-5-P were increased approximately 20 and 45 times, respectively, while that for dA-5-P was essentially unaltered (Fig. 2 *B*, *C*, *D*). Bessman has made similar observations independently.[21] As mentioned above, the kinase levels for

each of the four deoxynucleotides incorporated into phage T2 DNA reach values of about the same magnitude. By contrast, only traces of dC-5-P kinase were detected (Fig. 2 *E*). Furthermore, extracts of T2-infected cells were found to inhibit the dC-5-P kinase activity of normal cell extracts when equal amounts of infected and non-infected extracts were mixed. As will be described below, this inhibitory effect is due to an enzyme (dCTPase) which splits dCTP. By use of fluoride (8×10^{-3} *M*), which inhibits dCTPase more than 98 per cent, but the dC-5-P kinase by 15 per cent or less, it was possible to show that there was actually little or no change in the dC-5-P kinase levels upon infection. Extracts prepared by Method II showed the same kinase patterns after T2 infection.

Infection with phage T5, which contains cytosine rather than HMC, showed increased kinase activities for the four deoxynucleotides which are present in its DNA (Fig. 2 *B*, *C*, *D*, *E*). The 10-fold increase in dC-5-P kinase activity in the extracts of T5-infected cells may be contrasted with the absence of any increase in this activity in the extracts of T2-infected cells.

Hydroxymethylating activity was first detected at 4 min after T2 infection, and, as predicted from the results of Flaks and Cohen,[7] was absent from T5-infected cell extracts (Fig. 2 *F*).

An Enzyme which Destroys Deoxycytidine Triphosphate (dCTPase).—The inhibitor of dC-5-P kinase that develops upon T2 infection has been identified as an enzyme which splits dCTP by removal of the terminal pyrophosphate group (Fig. 3). Extracts of uninfected cells have 1 per cent or less of the dCTPase activity observed in extracts of T2-infected cells; the level of dCTPase activity of T5-infected cells was the same as in normal cells. The relative insensitivity to fluoride of the dCTPase activity in normal cells makes it doubtful that it represents the same enzyme found in the T2-infected cells.

After partial purification of the dCTPase,[19] which reduced the level of an inorganic pyrophosphatase to 2 per cent of the dCTPase, it was demonstrated that the complete splitting of 0.92 μmole of dCTP was accompanied by the appearance of 0.74 μmole of inorganic pyrophosphate identified as Norit-nonadsorbable, acid-labile P and 0.15 μmole of orthophosphate. With the purified enzyme preparation, the rates of cleavage of cytidine triphosphate and ATP were less than 1 per cent of that of dCTP; the splitting of dHMC-TP was 1–2 per cent of that of dCTP. The K_m of dCTP for the enzyme is in the region of 10^{-6} *M*. In view of this high affinity for dCTP and of the high level of enzyme activity compared to the dC-5-P kinase in the extract (cf. Fig. 2 *E* and Fig. 3), it is reasonable to suppose that the dCTP concentrations in the infected cell are reduced to an extremely low level.

An Enzyme which Glucosylates the HMC of DNA.—An enzyme which transfers glucose from UDPG to DNA containing HMC was observed in extracts of T2-infected but not T5-infected or normal cells (Fig. 4). With the partially purified enzyme[19] UDPG could not be replaced by glucose, glucose 1-phosphate or glucose 6-phosphate (Table I); similar results were also obtained with the crude extracts. The reaction requires HMC-containing DNA, which for these experiments was enzymatically synthesized from dHMC-TP, dATP, dGTP, dTTP, purified polymerase, and primer DNA derived from any one of several sources (calf thymus, *E. coli*, phage T2, phage ϕX174[22]). When DNA enzymatically synthesized with dCTP in place of dHMC-TP, or when the glucosylated DNA from phage T2 itself

were used, no glucose fixation in DNA was detectable (Table 1). dHMC-5-P and dHMC-TP failed to substitute for HMC-containing DNA as glucose acceptors. For example, in an incubation mixture containing these three types of HMC compounds with 30-fold purified enzyme, the HMC-DNA fixed 46 per cent of the glucose of the UDPG, while dHMC-5-P and dHMC-TP fixed none (<0.5 per cent).

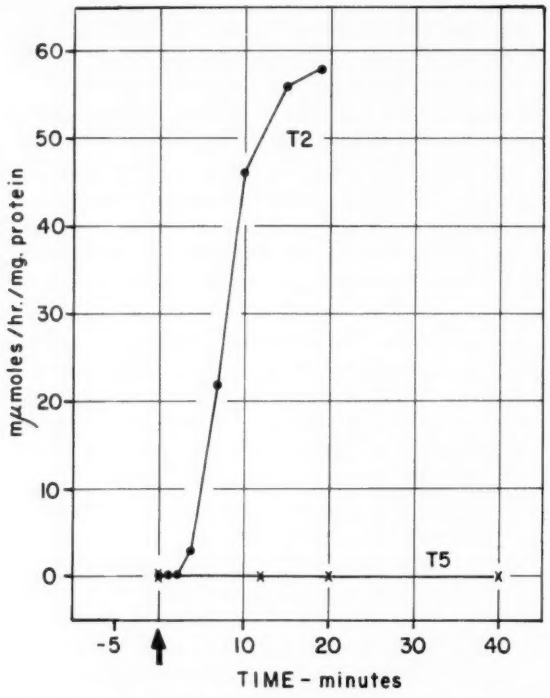

FIG. 4.—DNA-glucosylating enzyme levels before and after infection with phage T2 or T5. The arrow indicates the start of infection ("zero minutes," see Method I). The incubation mixtures (0.20 ml) contained: 10 mμmoles of UDPG labeled uniformly with C^{14} in the glucose residue (2×10^3 cpm per mμmole), Tris buffer, pH 7.5 (0.1 *M*), glutathione (0.02 *M*), DNA synthesized enzymatically (using thymus or phage T2 DNA as primer) and containing about 1 mμmole of HMC, and extract containing 10–50 μg of protein. After 15 min of incubation at 30°, the mixture was treated as in the "polymerase" assay of incorporation of a labeled deoxynucleotide into an acid-insoluble product (see Methods).

In the presence of an excess of HMC-DNA, the glucose of UDPG is transferred completely to the DNA. With an excess of UDPG and enzyme, the fixation of glucose in DNA reaches a limiting value, which is a function of the HMC-DNA present (Fig. 5). The number of glucose residues fixed in this experiment was approximately 60 per cent of the number of HMC residues in the added DNA. At this point it may be premature to regard the glucosylation limit observed with

TABLE 1

SPECIFICITY OF THE ENZYME WHICH GLUCOSYLATES DNA

Expt. No.	Conditions	Glucose fixed in DNA, mμmoles
1	Complete system (10 mμmoles of C^{14}-UDPG)	0.69
	Add C^{12}-UDPG (10 mμmoles)	0.65*
	Add C^{12}-glucose (200 mμmoles)	0.53
	Add C^{12}-glucose 1-P (200 mμmoles)	0.59
	Add C^{12}-glucose 6-P (250 mμmoles)	0.59
	Replace UDPG with C^{14}-glucose (10 mμmoles)	0.00
	Replace UDPG with C^{14}-glucose 6-P (12 mμmoles)	0.00
2	Complete system (DNA containing 0.46 mμmole of HMC)	0.28
	Replace HMC-DNA with cytosine-DNA (containing 0.75 mμmole cytosine)	0.00
	Replace HMC-DNA with T2 DNA (containing 2 mμmoles HMC)	0.00

The complete system had the composition and was treated as described in Figure 4, using a 30-fold purified enzyme, 0.3 μg in Expt. 1 and 1.5 μg in Expt. 2. In each case the DNA was glucosylated to its limit (see Fig. 5).

* The glucose fixed is calculated on the basis of the specific radioactivity of the UDPG after dilution of the C^{14} sample with C^{12}-UDPG.

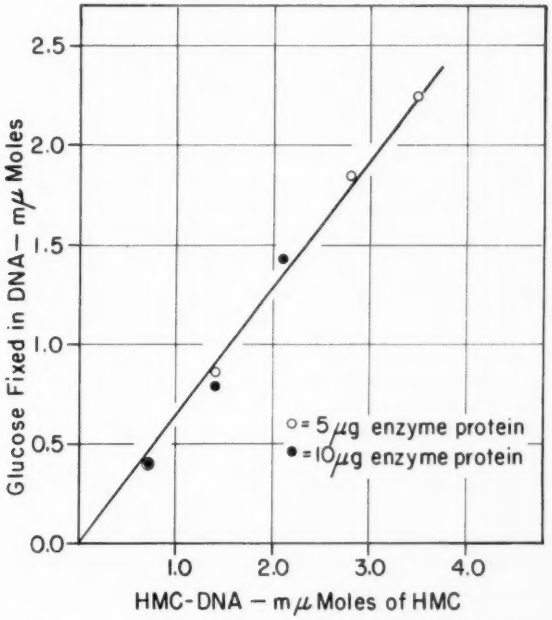

FIG. 5.—Limit of glucose fixation in DNA as a function of the amount of DNA added. The experimental details were as in Fig. 4, using the partially purified enzyme. The HMC-DNA was prepared with thymus DNA as primer.

a given sample of HMC-DNA as distinctive for the type of primer used in the enzymatic synthesis of the HMC-DNA. Further studies are required to determine how conditions of enzymatic polymerization, as well as the isolation of the DNA, may influence the glucose/HMC ratios obtained.

The C^{14}-glucose fixed in DNA was rendered acid-soluble by crystalline pancreatic deoxyribonuclease. When 52 per cent of the nucleotides were no longer acid-precipitable, 88 per cent of the glucose had been made acid-soluble. When the C^{14}-glucosylated HMC-DNA was digested to completion under conditions which Lehman has found[23] to yield a quantitative conversion of phage T2 DNA to 5′-mononucleotides, over 95 per cent of the radioactivity was found in the HMC deoxynucleotide fractions of the ion-exchange chromatogram.

Increase Rate of DNA Synthesis upon Infection.—DNA synthesis, measured by the standard assay,[2] but with dHMC-TP instead of dCTP, is increased 12-fold in extracts prepared 19 min after infection with T2 (Fig. 6). Little or no DNA synthesis can be measured in these extracts when dCTP replaces dHMCTP. However,

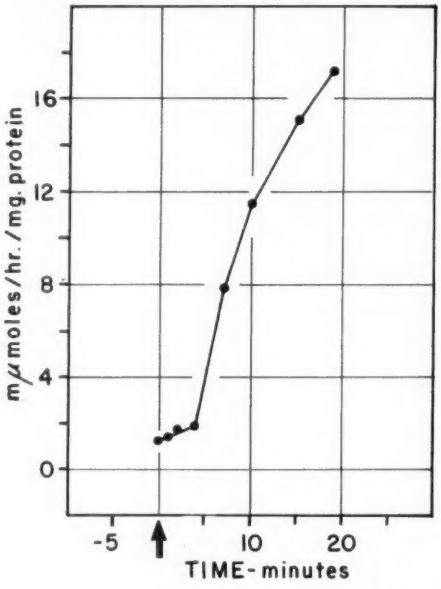

FIG. 6—DNA "polymerase" levels before and after infection with phage T2 with dHMC-TP as substrate in place of dCTP. The arrow indicates the start of infection ("zero minutes," see Method I). The incubation mixtures (0.30 ml) contained: 10 mμmoles each of dGTP, dATP, dTTP and dHMC-TP (C^{14}, 1×10^3 cpm per mμmole), Tris buffer, pH 7.5 (0.07 *M*), $MgCl_2$ (0.007 *M*), 2-mercaptoethanol (0.001 *M*), 0.04 ml. of "heated DNA" and sonic extract containing 10–50 μg of protein. The "heated DNA" was prepared by heating at 100° for 5 min, at pH 9.2, an extract of T2-infected cells; this preparation could be purified without loss of activity by treatment with ribonuclease, Norit, dialysis and precipitation with acid; it was inactivated by treatment with deoxyribonuclease. Further details of procedure were as referred to in Methods.

using 0.005 *M* F^- to inhibit dCTPase, DNA synthesis was elevated to levels near those observed in assays with dHMC-TP.

It should be emphasized that these measurements of rates of DNA synthesis with dHMC-TP were made with heated DNA as primer; when unheated DNA was used, there was no demonstrable increase in rate in the infected cell extracts. Heated DNA of phage T2 or calf thymus origin served as well as that used in Figure 6. The basis for this requirement for heated DNA with infected cell extracts requires further investigation.

DISCUSSION

In addition to the interest inherent in understanding the nature of viral infection of a cell, studies of the pathway of DNA synthesis in infected cells provide a means of testing and expanding our conceptions about the mechanism of DNA replication in normal cells.

Control of DNA Synthesis at the Level of Deoxynucleotide Phosphorylation.—It appears from studies with various analogues of the naturally occurring purines and pyrimidines[3] that an analogue in the form of a deoxynucleoside triphosphate is incorporated into DNA when its structure has the hydrogen-bonding properties of the base it replaces. For example, uracil and 5-bromouracil are both effective substitutes for thymine. Yet, uracil is never found in DNA[24] while bromouracil, when supplied to the cell, readily replaces thymine in the DNA.[25] The reason appears to be the absence of any mechanism for phosphorylating deoxyuridylate as contrasted with the availability of an enzyme for phosphorylating 5-bromodeoxyuridylate or 5-methyldeoxyuridylate (thymidylate).[3]

The present studies of phage-infected cells provide additional examples of control at the phosphorylation level. Earlier observations with 5-methyldeoxycytidylate[3] had suggested the lack of an enzyme for phosphorylation of a 5-substituted cytosine deoxynucleotide, and posed the problem of how 5-hydroxymethylcytosine deoxynucleotide becomes a substrate for phage T2 DNA synthesis. This problem seems to be solved by the development of a new enzyme after phage T2 infection of the cell. According to our studies, and those of Flaks and Cohen,[7] the synthesis of a compound novel for the cell is carried out by a new enzyme, the formation of which is presumably induced by the phage DNA. Another example of control at the phosphorylation level is provided by the device which appears to be responsible for the absence of cytosine in T2 DNA. For lack of a mechanism to eliminate the dCTP-synthesizing enzyme system, the evolution of an enzyme to destroy the dCTP provides an effective alternative.

Direct Substitution on DNA.—Genetic studies indicate that the glucose contents of DNA of phages T2, T4, and T6, and strains derived from them by recombination, are a fixed property resembling other phenotypic characters of these phages.[9, 10] It appears plausible, therefore, that the precise arrangement of these glucosylated HMC residues in the DNA may be a source of genetic information, and insight into the nature of the replication of these types of phage DNA would clearly be of considerable value.

The incorporation of a fixed proportion of non-, mono-, and polyglucosylated derivatives of dHMC-TP during the polymerization of the DNA chain is difficult to conceive because it is likely that these derivatives would all behave similarly in hydrogen-bonding to guanine. It becomes even more difficult to conceive the incorporation of these various HMC residues in a precise sequence in DNA on the basis of selection of the triphosphate derivatives. The existence of an enzymatic system for direct glucosylation of DNA offers an alternative which seems to circumvent these difficulties. At this stage, the information is still too fragmentary to determine whether this glucosylating enzyme, and perhaps an additional one for polyglucosylation, will be sufficient to explain the replication of various phage DNA's. It is apparent that further studies with T4- and T6-, as well as T2-infected cell enzyme systems are essential.

Superficially analogous to the partial glucosylation of the HMC residues in phage DNA is the partial methylation of the cytosine residues in certain plant and animal DNA's, such as wheat germ and calf thymus.[24] In the light of our findings, it would be interesting to look for an enzymatic mechanism for direct methylation of DNA in these tissues.

Kinetics of Enzyme Development and Enzyme Reactions.—The temporal pattern of development of all the enzymes studied in this report that were either "new," or the levels of which were significantly raised, was similar. The first traces of change were apparent at four minutes after T2 infection and about ten minutes after T5 infection. While the precision of measurement of these time intervals is not great, it is clear that there is a time lag before significant levels of these enzyme activities appear. Several groups of investigators[26, 27, 28] have shown that 5-methyltryptophan and chloramphenicol administered to cells at levels sufficient to inhibit protein synthesis also blocked DNA synthesis when given before or up to about 5 min after infection. When given 10 min or later following T2 infection, the inhibition of protein synthesis had only a small or even no effect on the rate of DNA synthesis. It seems reasonable to consider that the time lag we have observed may be related to the chloramphenicol-sensitive interval required for development of the enzymatic machinery for DNA synthesis, as well as other protein components vital to virus information. There have been indications of non-phage protein synthesis immediately upon infection[29] and it remains to be determined what fraction of this protein can be identified with the enzymes described here.

The multiplicity of enzyme changes described in this study along with the findings on the hydroxymethylating[7] and deoxyuridylate-methylating[30] enzymes, are all directly related to DNA synthesis. Enzyme measurements related to other metabolic pathways in infected cells have as yet disclosed few significant changes.[31] It would be surprising if further exploration of the phage-infected cell failed to reveal additional examples of new or augmented enzyme activities related to the requirements imposed by rapid phage synthesis.

In view of the pitfalls inherent in assaying the level of an enzyme activity in a cell extract, let alone in the cell itself, a detailed evaluation of the various enzyme values in terms of virus DNA synthesis does not seem warranted. However, it is interesting to note that the rates of the kinases and the glucosylating enzyme are all greater than that found for the DNA polymerizing activity and, further, that the increase in the latter activity over levels found in uninfected cell extracts is about the same as the increase in DNA synthesis in whole cells following T2 infection. Also it is remarkable that the dA-5-P kinase activity (very likely identical to adenylate kinase) is about 10 times that of the other deoxynucleotide kinases in the uninfected cell extract and does not change upon T2 infection, while after infection the other kinases and hydroxymethylase reach levels comparable to the dA-5-P kinase. Finally, it is noteworthy that the dC-5-P kinase activity does not increase upon T2 infection but remains at the relatively low level observed in the uninfected cell, whereas it increases about 10-fold upon T5 infection.

References to increases in level of a preexisting enzyme carry no implication that the additional enzyme activity is identical to the old or even that more enzyme has been synthesized "*de novo*." To resolve this important point, it will be necessary to characterize isolated preparations of the normal- and infected-cell enzymes and to establish by tracer techniques that the enzymes developed after infection have, like induced enzymes,[32] been synthesized from the amino acid pool.

Control of DNA Synthesis by the Nature of the DNA Primer.—Perhaps the most

interesting question regarding T2 infection of *E. coli* is how the T2 DNA seems to preempt from the host DNA the primer function for the polymerase system. The present studies can explain why DNA synthesized by the phage T2-*E. coli* system contains hydroxymethylcytosine rather than cytosine, but not why the base composition is that of phage rather than that of host DNA. An observation reported in this paper which may bear on this question is the dependence of the augmented polymerase activity of the infected cell extracts on a physically altered DNA primer. When "native" calf thymus or phage T2 DNA was used, the polymerase values of infected and normal cell extracts were similar. However, with DNA samples heated at pH 9, the values for dHMC-TP incorporation with infected cell extracts were increased 12-fold, while the normal cell values were unaffected or even depressed. It is not clear as yet whether infection has resulted in formation of a new type of polymerase or in an increase of the normal polymerase and new factors which modify it. Further studies to clarify this question may lead to a better insight into how the injected phage DNA proves to be the chosen primer for this system.

SUMMARY

1. Extracts of *E. coli* infected with bacteriophage T2 have been shown to contain three enzymes which are undetectable in extracts of uninfected or in T5-infected cells. These are: (*a*) an enzyme which phosphorylates hydroxymethyldeoxycytidine 5-phosphate, leading to the synthesis of the triphosphate, (*b*) an enzyme which removes the terminal pyrophosphate group specifically from deoxycytidine triphosphate, and (*c*) an enzyme which transfers glucose from uridine diphosphate glucose directly to the hydroxymethylcytosine of certain DNA's.

2. These new enzymes can account for (*a*) the availability of the triphosphate of hydroxymethyldeoxycytidine for the enzymatic synthesis of T2 DNA, (*b*) the absence of deoxycytidylate from T2 DNA, and (*c*) the presence of glucose on a fixed fraction of the hydroxymethylcytosine residues in DNA.

3. The DNA-polymerizing enzyme assayed with hydroxymethyldeoxycytidine triphosphate in place of deoxycytidine triphosphate reveals about a 12-fold increase in activity after T2 infection. Increases, after T2 infection, in the levels of thymine and guanine deoxynucleotide phosphorylating enzymes (by 20–45 fold) bring their activities up to the level of the adenine deoxynucleotide phosphorylating enzyme which is unchanged; the level of the cytosine deoxynucleotide phosphorylating enzyme remains at a low level.

4. After T5 infection, levels of the thymine, guanine, and cytosine deoxynucleotide phosphorylating enzymes increase by 10–40 fold, bringing their activities up to the level of the adenine deoxynucleotide phosphorylating enzyme, which increases about 2-fold.

5. The new enzymes and the increases in level of the enzymes occurring in normal cells are first detectable about 4 min after infection with phage T2 and about 10 min after infection with phage T5. These results are consistent with previously published studies which have indicated with the use of inhibitors of protein synthesis that viral DNA synthesis requires a preliminary period of protein synthesis.

* This investigation was supported by research grants from the National Institutes of Health of the Public Health Service and the National Science Foundation.

† National Science Foundation Predoctoral Fellow.

‡ Fellow of the National Foundation.

[1] The abbreviations used in this report are: cpm, counts per minute; ATP, adenosine triphosphate; dA-5-P, deoxyadenosine 5′-phosphate; dC-5-P, deoxycytidine 5′-phosphate; dG-5-P, deoxyguanosine 5′-phosphate; dHMC-5-P, hydroxymethyldeoxycytidine 5′-phosphate; dT-5-P, deoxythymidine 5′-phosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; dHMC-TP, hydroxymethyldeoxycytidine triphosphate; dTTP, deoxythymidine triphosphate; DNA, deoxyribonucleic acid; HMC, 5-hydroxymethylcytosine; UDPG, uridine diphosphate glucose; Tris, tris-(hydroxymethyl)aminomethane; KPO_4, potassium phosphate buffer; PRPP, 5-phosphoryl α-D-ribofuranose 1-pyrophosphate; E_m, molar extinction coefficient.

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[19] For large-scale enzyme preparations, 50-liter cultures at the late exponential phase (2×10^8 cells/ml), in modified M-9 medium (see Methods) but lacking $CaCl_2$, were treated with 3–4 $T2r^+$ per cell and 10 min later with 50 γ of chloramphenicol per ml. The culture was then chilled to 0° with ice over a 10-min period and harvested by centrifugation. The initial steps in the purification of the dHMC-5-P kinase, dCTPase, glucosylating enzyme, and hydroxymethylating enzyme were the same. Sonic extracts, prepared in 5 volumes of 0.05 *M* glycylglycine buffer, pH 7.0, were centrifuged and the supernatant fluid diluted with buffer to contain 10 mg of protein per ml. Streptomycin sulfate (5 per cent solution), equal to 0.3 volume of the diluted extract, was added; the supernatant fluid collected after centrifugation contained 3 to 4 mg of protein per ml. It was adjusted to pH 8 with KOH and applied to a diethylaminoethylcellulose (Brown Co.) column [Peterson, E. A., and H. A. Sober, *J. Am. Chem. Soc.*, **78,** 751 (1956)], equi-

librated with 0.02 *M* KPO_4, pH 8.0, containing 0.01 *M* 2-mercaptoethanol. Linear gradients were applied and the purifications obtained are expressed relative to the supernatant fluid of the sonic extract; all solutions contained 0.01 *M* 2-mercaptoethanol. Glucosylating enzyme: gradient of 0.08 to 0.32 *M* NaCl in 0.02 *M* KPO_4, pH 8.0–30× purification; dCTPase: gradient of 0.08 *M* to 0.32 *M* NaCl in 0.02 *M* KPO_4, pH 8.0–24× purification, rechromatographed with a gradient of 0.06–0.32 *M* KPO_4, pH 6.5–85× purification; dHMC-5-P kinase: gradient of 0.06 to 0.32 *M* KPO_4, pH 6.5–20× purification; hydroxymethylating enzyme: gradient of 0.3 *M* KPO_4, pH 6.5 to 1.0 *M* KPO_4, pH 6.1–230× purification. Fractions containing 2-mercaptoethanol were not frozen. Active fractions were concentrated by precipitation with solid $(NH_4)_2SO_4$. Further details of the purification procedures will be published elsewhere.

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*PURIFIED MUSCLE PROTEINS AND THE WALKING RATE OF ANTS**

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Communicated by Donald D. Van Slyke, April 24, 1959

Muscular contraction represents a fascinating problem in the conversion of chemical energy into mechanical work. To elucidate the mechanisms at a molecular level, however, requires that the enzymatic activities responsible for muscular contraction be separated from the many other activities present in a complex cell. A system which apparently isolated the contraction process was found by Szent-Gyorgyi in 1942 when he observed that actomyosin obtained from rabbit muscle would "superprecipitate" on addition of adenosine triphosphate (ATP).[1] Since then, Weber and others[2] have studied the extracted muscle fiber and a number of more or less oriented actomyosin threads. That such systems are good models

was indicated by a number of indirect lines of evidence: (*a*) The protein, actomyosin, which superprecipitates comprises 70 per cent of the extractable protein of muscle tissue.[1,2] (*b*) Fiber and thread models show a number of mechanical properties, e.g., rate of shortening, extent of shortening, etc., very similar to those observed in isolated muscle tissue from rat diaphragms, frog legs, etc.[2,3] (*c*) The superprecipitation phenomenon occurs under physiological conditions as far as temperature, pH, and ionic strength are concerned.[1-3]

Using extensively purified preparations of actomyosin, myosin and actin, studies

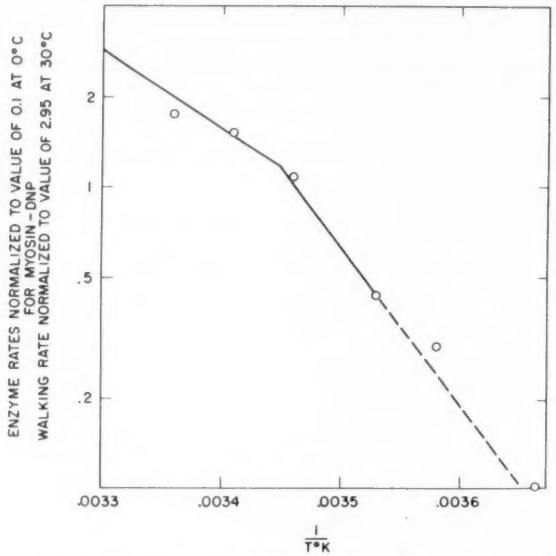

FIG. 1—Effect of temperature on the ATPase activity of myosin in the presence of 0.01 *M* dinitrophenol and on the walking rate of ants. Solid line obtained from Crozier[7] representing measurements on hundreds of ants (Shapley[6]). Points (○) represent rate of hydrolysis of ATP by myosin in presence of DNP. The ant walking rate was normalized to a value of 2.95 at 30°C representing a rate of 3.05 cm/sec. The DNP-myosin (open circles dotted line) curve, for purposes of comparison, was normalized to a value of 0.1 at 0°C, at which temperature 0.002 μ mole of inorganic phosphate was released from ATP per min per mg protein. Solution for myosin ATPase measurements contained: 0.1 *M* KCl, 0.01 *M* $MgCl_2$, 0.05 *M* tris, pH 7.3, 0.005 *M* ATP, 0.01 *M* DNP.

in our laboratory with isotopic techniques have indicated that an intermediate is formed during the hydrolysis of ATP by superprecipitating actomyosin.[4] Moreover it could be shown that these isotopic phenomena were associated with the contractile proteins themselves and not with contaminants absorbed during the isolation process.[4] The increasing success of the *in vitro* system in satisfying the biochemical demands of purity inevitably led to a desired for more assurance that the system was indeed a true model for muscular contraction. While the above cited evidence was strong, it was of necessity a somewhat indirect correlation of the purified system

to muscle tissue already severed from its natural source. It was desirable, therefore, to obtain a direct link between the enzymatic activity of the purified muscle proteins and the process of muscular contraction in an intact, living system.

An opportunity for a correlation of this sort was suggested by some temperature studies on myosin ATPase activity. It was observed[5] that dinitrophenol had a marked effect on the shape of the rate-temperature profile and on the kinetic constants obtained at various temperatures when ATP was hydrolyzed in the presence of myosin. In the absence of dinitrophenol the Arrhenius plot was linear with an activation energy of circa 12 kcal over the entire temperature range (0°–30°). In the presence of dinitrophenol, the Arrhenius plot has a linear portion with an activation energy of 12 kcal in the neighborhood of 25° and a linear portion with an activation energy of approximately 24 kcal in the neighborhood of 5°. The transition from the 12-kcal process to the 24-kcal process occurs rather rapidly in the neighborhood of 16°. An exceedingly provocative fact about this curve was its similarity to the temperature dependence of the walking rate of ants studied by Shapley[6] and interpreted by Crozier[7] in the 1920's (cf. Fig. 1). While dinitrophenol is not present *in vivo*, indications have been obtained by Chappell and Perry[8] and in the work in this laboratory[5] that actin had an effect on myosin ATPase very similar to that of dinitrophenol. A logical deduction from these findings, therefore, was that the actomyosin temperature curve would show a temperature dependence similar to the ant walking rate and hence establish the desired relation between the superprecipitation phenomenon and an *in vivo* process.

The data in Figure 2 show the results of such a study on actomyosin. Actomyosin was obtained by isolating it directly from rabbit skeletal muscle or by reconstituting it from actin and myosin obtained separately from rabbit skeletal muscle.[1] The rate of hydrolysis of ATP added to this purified protein system was measured over the temperature range from 30° to 0°. Since the hydrolysis of ATP by purified myosin preparations has been shown to undergo an initial rapid burst followed by a slower linear portion[9] care was taken to compare the rates of hydrolysis at comparable portions of the hydrolysis curve. In the data shown in Figure 2 these were all taken in the second phase or linear portion. Comparison of the rates in the first initial burst, however, gave the same over-all dependence. The ionic strength of the solution was physiological, and by maintaining this low ionic strength it was possible to demonstrate that superprecipitation occurred over the entire temperature range. Not only was this observed visually but aliquots of the supernatant fluid after obvious agglomeration of the muscle proteins were taken and assayed for protein by trichloracetic acid precipitation. In no case was any significant amount of protein found in the supernatant fluid. This was an important condition of the experiments since at higher ionic strength, ATP causes dissociation of actin and myosin.

The curves derived from these data are approximately linear at the higher and lower temperatures and the activation energies calculated from the slopes of the appropriate tangents are 12 kcal and 25 kcal. A rather rapid transition from one linear phase to the other occurs at about 16°. For purposes of comparison, the data for the ant walking and for the myosin and actomyosin catalyzed hydrolysis are plotted on the same graph. The similarity between the raw data, the calculated activation energies and the transition temperature is striking. One is led to con-

clude that the rate limiting reaction in the walking rate of ants is the hydrolysis of ATP catalyzed by actomyosin.

The suggestion that the rate limiting step in a physiological process might be identified by its temperature dependence was originally made by Crozier.[10] Crozier embodied this assumption in a hypothesis which had several other features. Since some of these added features have been shown to be incorrect the Crozier hypothesis

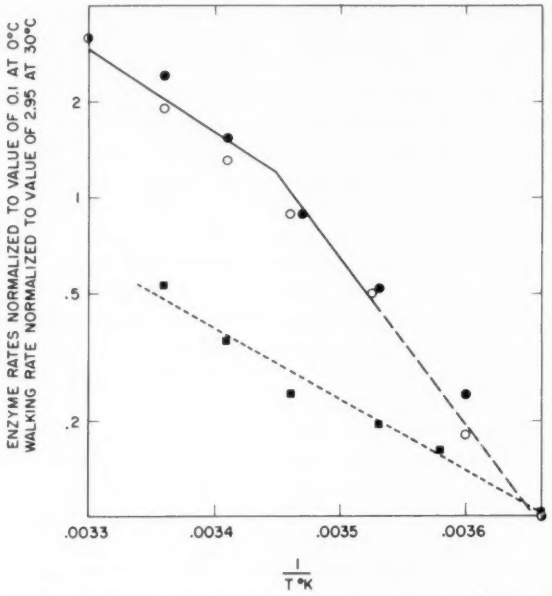

FIG. 2.—Effect of temperature on the ATPase activity of myosin and actomyosin and on the walking rate of ants. All enzymatic rates put on the same arbitrary scale, for comparison, by normalizing to a value of 0.10 at 0°C. Observed values, 0°C, for "natural" isolated actomyosin (●) 0.0015 μmoles P_i/min/mg protein; reconstituted actomyosin (○) 0.0013 μmole P_i/min/mg protein; myosin (■) 0.0010 μmole P_i/min/mg protein. Curve for walking rate of ants (solid line) taken from Crozier[2] (same as Fig. 1) was superimposed by normalizing to a value of 2.95 at 30°C. Actual measured rate of walking at 30°C was 3.05 cm/sec; at 9°C (lowest temperature studied) rate was 0.44 cm/sec. For enzyme studies with actomyosin, the solution had the following composition: 0.025 *M* KCl, 0.005 *M* $MgCl_2$, 0.025 *M* tris (hydroxymethyl) amino methane, pH 7.3, 0.0023 *M* ATP; with myosin conditions were those given in Fig. 1, but without DNP.

as a whole has been largely ignored or rejected. The failure to draw a warranted conclusion can, however, be as deleterious to science as a hasty jumping to an unwarranted one. Hence it may be well to re-examine the Crozier hypothesis in the light of more recent work on enzyme action. Crozier proposed that (*a*) in many physiological processes a single enzymatic reaction was the rate-limiting or "pacemaking" step, (*b*) that this pacemaking step usually controlled the rate of the physiological process over a sufficient temperature range to obtain a characteristic activa-

tion energy, (*c*) that at certain temperatures an abrupt change ("break") occurred from one pacemaking step to another and (*d*) that the rate limiting step in the physiological process could be identified by the activation energy of a specific reaction.

This hypothesis was subject to numerous criticisms, the most important of which are (*a*) that the change from one rate limiting process to another could not be as abrupt as proposed by Crozier[11-14] and (*b*) that the inaccuracy of the data will allow many apparent coincidences which do not reflect a true correspondence. Regarding the abrupt changes, the arguments are convincing that on the one hand the physiological data do not warrant a discontinuous change as Crozier postulated, and that. on the other hand, a shift from one rate limiting enzymatic step to another would not give as rapid a change as was, indeed, observed. Regarding the identification of the enzyme activity it soon became apparent that many enzymes had rather similar activation energies. This would require great accuracy in the data to obtain identification but many of the physiological processes were measured inaccurately and over a very limited temperature range.

With these pitfalls clearly in mind, let us examine the present case. In the first place the data for the *in vitro* enzyme and for the walking rate of ants are quite accurate. Shapley reports that they were able to predict the temperature within 1° after timing ten to twenty ants. In the second place a large temperature range is available because ants are cold-blooded and measurements of an *in vivo* process could be made at temperatures down to 9°C. As a result a quantitative identification between the physiological process and the purified enzyme are possible over a wide temperature range. The agreement is within experimental error at every temperature. This provocative correspondence is not obtainable with any other enzyme whose temperature dependence is known.

The fact that a single enzyme could show such a biphasic temperature dependence was originally demonstrated by Sizer, who suggested that the "break" in the Arrhenius plot was caused by a change in the configuration of the protein.[15] This conclusion is significant in two ways as far as the Crozier hypothesis is concerned. Firstly, it presents a plausible explanation for the rather abrupt change in activation energy. It does not change as abruptly as pictured by Crozier; in all cases a smooth curve can be drawn in the neighborhood of the transition. On the other hand it can change more abruptly than would be calculated for the shift from one rate limiting reaction to another. Since the activation energies for protein configuration changes vary considerably it would be expected that considerable range in the abruptness of the transition would occur. This is observed to be the case. In some cases no change or a very gradual one is observed; in others, the entire shift appears to be accomplished within a few degrees centigrade. Secondly, the protein change means that the identification of the rate limiting step in a physiological process will be simplified in some cases and the present case appears to be one of them. Thus, at higher temperatures actomyosin, myosin, and the walking ants all show the same activation energy of *ca.* 12 kcal. No choice between myosin and actomyosin would be possible in this region. However, by including the data to 0°, it is seen that the ant walking rate shifts to a process having an activation energy of 25 kcal and that the transition occurs rather suddenly in the region of 16°. With these added characteristics only actomyosin coincides over the entire temperature

range. The protein change, therefore, contributes added marks of identification which make a coincidental correspondence less likely.

A re-evaluation of the Crozier hypothesis, therefore, indicates that it is faulty in relation to the abruptness of the transitions and the reason for the transitions. However, on its two most important postulates, i.e., that certain physiological processes have a single rate limiting enzymatic step and that the rate limiting step can be identified by comparison of Arrhenius plots, it appears to be correct. The number of systems providing enough characteristics and sufficient accuracy to allow identification may be very small, but it appears that the walking rate of ants is one system in which such an identification is possible.

A number of additional conclusions can also be derived from these data. In the first place, the similarity of the dinitrophenol-myosin curve and the actomyosin curve supports the conclusion[4,6] that actin and dinitrophenol have some similar effects on the myosin-catalyzed hydrolysis of ATP. Clearly they are not identical in all respects since the dinitrophenol-myosin system does not contract but the conclusion that the activating effects of these two quite different reagents have similar features is supported by the close correspondence of the temperature curves. In the second place, it is to be noted that the parameter which correlates with the walking rate of ants is the rate of hydrolysis of ATP. By inference, therefore, the hydrolysis rate is proportional to contraction. However, it should be added that the correlation between superprecipitation and contraction is complicated by reactions which seem to be analogous to rigor. Therefore, while rate of superprecipitation appears to be a function of the primary event in contraction, the precise nature of the relationship has not yet been worked out. Thirdly, it is to be noted that the most purified model system we have for muscular contraction, the purified actomyosin system, has been correlated with a true *in vivo* process. This not only bolsters the arguments that the molecular events in the highly purified system are those occurring during muscular contraction but also adds further support to the argument[2,3] that the intermediate models, e.g., the fibers, fibrils, and intact cell systems, are also good models for the *in vivo* process. Fourthly, it is to be noted that the same temperature dependencies are observed for isolated actomyosin as for actomyosin reconstituted from purified actin and purified myosin. This further establishes the similarity of these two different preparations and shows that whatever orientation is necessary for the functional interaction between individual units of actin and myosin can be achieved by reforming the gel or thread from them under the appropriate conditions. Thus both the superprecipitation process, and, by the arguments presented above, the contraction process, must be a consequence of the chemical properties of actin and myosin. Whatever macromolecular orientation occurs *in vivo* must be merely to obtain the maximum efficiency for unidimensional contraction and does not change the chemistry of the molecular events.

Summary.—It is observed that the temperature dependence of the walking rate of ants is strikingly similar to the hydrolysis of ATP by superprecipitating actomyosin over the entire temperature range of 30°C to 10°C. From this it is concluded that the rate limiting step in the walking rate of ants is probably the hydrolysis of ATP by actomyosin in the *in vivo* system. Moreover, it is felt that this correspondence between the clearly *in vivo* process and the highly purified protein system supports the argument that the chemical events in the purified system are the

same as those occurring during muscular contraction.

* Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

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*A FUNCTIONAL DISORDER OF MUSCLE ASSOCIATED WITH THE ABSENCE OF PHOSPHORYLASE**,†

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Communicated by Carl F. Cori, April 22, 1959

Introduction.—The present report deals with the biochemical identification of the defect in a recently encountered muscle disorder, characterized by a rapid exhaustion of otherwise mechanically and physiologically normal muscle, which exhaustion could be prevented by the intravenous administration of lactate, glucose or fructose. The case, which has some resemblance to the one reported by McArdle (1951), will be described extensively elsewhere (Pearson, Rimer and Mommaerts, 1959).

The observed peculiarities gave the impression that, while the capacity for resting or low-activity metabolism is sufficient, a block exists preventing the transition from rest to higher rates of activity metabolism. As long as only the preventive effect of lactate was known, we thought, as McArdle had concluded for other reasons, that this restriction might resemble that in iodoacetate poisoning and consist of a defect in phosphoglyceraldehyde dehydrogenase. The subsequent observation that glucose (and also fructose, Pearson, 1959), can likewise alleviate the symptoms pointed to a block closer to the phosphorolysis of glycogen.

The studies reported herein show that the disease is characterized by a lack of phosphorylase combined with a considerable storage of glycogen.

Materials and Methods.—Tissue material was obtained from the patient (D. G.), a 19-year-old male, in the fasting state by biopsy from the lateral aspect of the right upper thigh under local anesthesia. Immediately from the operating table, separate tissue fragments were immersed in a large Dewar flask with isopentane at −180°C. They were then transferred to liquid nitrogen, coarsely powdered, and stored in a −70°C cryostat. Samples taken for analysis were finely powdered at −180°C, and extracted at 0°C with 0.001 *M* aqueous ethylenediaminetetraacetate (with or without fluoride) for the enzyme assays; with 0.3 *N* perchloric acid for the analysis of soluble metabolites; or with 3 volumes of 30 per cent KOH at 100° for the determination of glycogen. The further methodology for each of these studies will be described under the respective headings.

Results.—Glycogen: Determinations of glycogen were performed by the method of Good *et al.* (1933), using the anthrone reagent as described by Hassid and Abraham (1957). The results are shown in Table 1. It is seen that the resting

TABLE 1

DETERMINATION OF GLYCOGEN IN DISEASED (D.G.) AND NORMAL HUMAN MUSCLE

Mgs. Glycogen Per Gram Tissue	
D.G.	Normal
41.2	9.2
37.5	9.4

glycogen value is in marked excess over the accepted standard values, 10 to 20 mg glycogen per gram. A sample taken from normal human thigh muscle gave a figure in the expected range. In keeping with the analytical results, it was found that histochemical staining (cf. Pearson, Rimer and Mommaerts, 1959) applied to a biopsy sample sectioned in the frozen state, showed excessive amounts of glycogen in a diffuse granular form.

Glycolysis in tissue extracts: In minced muscle suspended or extracted in buffer, glycolysis occurs at a fairly reproducible rate which provides some measure of the glycolytic capacity. Frozen muscle powder was homogenized in 0.001 *M* phosphate buffer of pH 7.4. The centrifuged extracts were incubated anaerobically and analyzed for lactate by the method of Barker and Summerson (1941) after deproteinization with trichloroacetic acid. Typical results are those of Figure 1 obtained on extracts to which nicotinamide was added. Similar results have been observed without this addition and in whole homogenates. It is seen that in the diseased muscle glycolysis from endogenous glycogen is negligible. Added glycogen is not glycolyzed either, but glucose-1-phosphate and glucose-6-phosphate raise the activity to quite normal levels. This would indicate that, but for the initial step of glycogenolysis, the glycolytic enzyme system is sufficient to sustain significant rates of metabolism. The glycolytic rate in diseased muscle on addition of hexosephosphates is actually two or three times as high as comparable rates obtained with several biopsy samples of normal muscle. However, in view of the many factors which influence the rate of glycolysis in extracts (Neufach and Melnikova, 1958), it is not justified to say without further investigation that the diseased muscle is richer than normal in some rate limiting enzyme. The major result is qualitative:

there is no glycolysis with glycogen, but seemingly normal magnitudes of activity with glucose-1-phosphate and other intermediates.

These experiments suggest that glycolysis in the diseased muscle is limited either by absence of phosphorylase, or by an inadequate transformation of phosphorylase *b* to *a*.

Determination of phosphorylase a and b: Direct tests for the presence or absence of phosphorylase were performed by extracting powdered frozen muscle with 4 volumes of water containing 0.001 *M* ethylenediaminetetraacetate (EDTA) (cf. Krebs and Fischer, 1955a) with or without 0.1 *M* fluoride for 20 min. Assays for

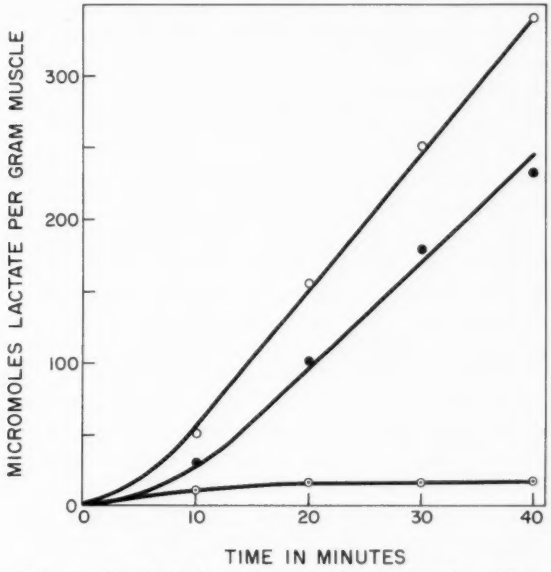

FIG. 1.—Glycolysis in extracts of diseased muscle, with no addition (⊙), glucose-6-phosphate (○) or glucose-1-phosphate (●). Composition of medium: 0.3 ml 0.024 *M* K-phosphate buffer pH 7.6; 0.3 ml 0.5 *M* K HCO_3; 0.1 ml 0.01 *M* Na-K-ATP; 0.2 ml 0.2 per cent DPN; 0.2 ml 0.1 *M* Mg Cl_2; 30 μ moles of substrates; 0.4 ml 0.025 *M* nicotinamide; water to 3 ml final volume, including 0.5 ml tissue extract, corresponding to 50 mg muscle. Temperature 37°C.

phosphorylase *a* and *b* were done according to Illingworth and Cori (1953). As shown in Table 2, the pathological muscle contained no detectable amounts of either phosphorylase *a* or *b*. Nor could pyridoxal-5-phosphate (cf. Illingworth *et al.* 1958), 3.8 μ moles added during extraction and sometimes also during the pre-incubation, induce such activity. Histochemical phosphorylase tests according to Takeuchi (1958) were also completely negative, contrary to comparably treated samples from normal human muscle (cf. Pearson, Rimer and Mommaerts, 1959). The absence of detectable phosphorylase *b* could not be ascribed to abnormally rapid deamination of the adenylic acid added to the assay, since no excessive adenylate deaminase content was demonstrable. Disappearance of a sizable fraction

TABLE 2

ANALYTICAL DATA ON ASSAY FOR PHOSPHORYLASE *b* AND *a* IN DISEASED (D.G.) AND NORMAL MUSCLE

Muscle samples (162.6 and 161.3 mg, respectively) were extracted with 0.4 ml 0.001 *M* EDTA; of the extracts, 0.1 ml samples were diluted 20-fold with 0.04 *M* Na-glycerol phosphate-0.03 *M* cysteine at pH 6.0; 0.4 ml of this solution was incubated with 0.2 ml of 4 per cent glycogen at 30°C for 20 min. At zero time, 0.2 ml of 0.064 *M* glucose-1-phosphate was added, with or without 0.004 *M* AMP. Aliquots of 0.2 ml were taken at intervals for analysis. For the phosphoglucomutase correction test, a 0.1-ml aliquot was used.

	D.G.		Normal	
Phosphorylase Assay	Without AMP	With AMP	Without AMP	With AMP
γ P at zero time in aliquot	6.6	7.3	6.5	6.9
γ P per aliquot formed				
5 min	+0.3	−0.1	+0.1	+13.2
10 min	+0.4	+0.1	+0.1	+15.1
15 min	...	...	+0.9	+16.8
Phosphoglucomutase Correction Test				
γ P at 10.5 min	26.2	26.3	24.1	25.2
Expected from G-1-P hydrolysis		26.3; 26.2		

of the added AMP would be possible during the course of the test, but the activity was not raised by fluoride (cf. Krebs and Fischer, 1955b). Phosphorylase assays were likewise negative when performed at lesser dilution of the enzyme, and at incubation times as long as 1 hour at pH 6.2. Addition of crystalline phosphorylase *a* to the reaction mixture resulted in complete recovery of its activity.

Phosphorylase kinase and PR enzyme: The absence of phosphorylase might be unique, or might be associated with an absence of its activating and deactivating enzymes. Hence, determinations of phosphorylase kinase and of phosphorylase phosphatose (PR enzyme) were performed. The former enzyme was assayed following the procedure of Krebs and Fischer (1956), and an activity of 683 units per gram of muscle was found. The latter was assayed according to Keller and Cori (1955) with a yield of 3870 units per gram of muscle. These amounts are significant, illustrating that the absence of phosphorylase is sharp and specific, and is not associated with generalized defects of biologically related enzymes.

Determination of UDPG-glycogen transferase: For reasons set forth in the *Discussion*, it was apparent that the formation of glycogen could not take place by reversal of the phosphorylase reaction, but must occur by the UDPG pathway first suggested by Leloir and Cardini.

UDPG-glycogen transferase was determined by the method of Hauk *et al.* (1959), such that a sample of frozen muscle was homogenized with nine volumes of 0.02 *M* Tris-0.001 *M* EDTA buffer at pH 7.5 for 3 min at top speed in a Virtis homogenizer, and an aliquot of the uncentrifuged homogenate was assayed by 15 min incubation at 30°C in a reaction mixture containing 0.001 *M* UDPG, 1 per cent glycogen, 0.003 *M* $MgCl_2$ and 0.04 *M* Tris buffer, pH 7.2. An activity of 6 transferase units (Hauk *et al.*, 1959) per gram of tissue was found, which is in the normal range previously found in well preserved tissues.

Chemical nature of deposited glycogen: A sample of glycogen was isolated and subjected to the combined action of phosphorylase and amylo-1,6-glucosidase (Illingworth, Larner and Cori, 1952). The glycogen was completely digested and had a normal endgroup percentage of 7 per cent. When treated with phosphorylase free of amylo-1,6-glucosidase, 41 per cent of the glucose residues present in the

glycogen were liberated, showing that the length of the outer chains was likewise within the normal range (Illingworth and Cori, 1952). Thus, the glycogen formed in the absence of phosphorylase, but presumably in the presence of branching enzyme, had in every respect a normal structure.

Determination of some phosphorus compounds and other metabolites: Analyses were performed for some of the major phosphate-containing metabolites on perchloric acid extracts, using the standard methods of this laboratory (Mommaerts, 1957). None of the comparisons between the pathological samples and normal muscle are accurately valid, since during the biopsy procedures no precautions could be taken to have all specimens in the same physiological condition. Also, not enough information is available about the spread of normal values. With these restrictions in mind, it can be said that the results (Table 3) are not indicative of

TABLE 3

DETERMINATIONS OF SOME METABOLITES IN DISEASED AND NORMAL HUMAN MUSCLE

	Micromoles per gram of tissue	
	D.G.	Normal
Free creatine[1]	6.4	9.2
Phosphocreatine[2]	16.0	13.3
Inorganic P[3]	7.4	7.0
Hexosemonophosphate[4]	0.22	0.35
Hexosediphosphate[5]	0.25; 0.22	0.48; 0.30
Lactate[6]	1.1; 1.2	2.7; 2.9
ATP[7]	5.55	5.36
Total nucleotide[8]	6.15	6.17

[1] Methods (cf. Mommaerts, 1957): Diacetyl method; [2] *Ibid.*, increase upon hydrolysis; [3] Isobutanol extraction method; [4] Slater's method B; also separate determination of glucose-6-phosphate with glucose-6-phosphate dehydrogenase and TPN after acid hydrolysis of ATP; [5] Including triosephosphates, according to Slater's method A; [6] Barker and Summerson's method; [7] Reduction of TPN with glucose-6-phosphate dehydrogenase and hexokinase; [8] Ultraviolet absorption calculated for adenine nucleotides.

any significant change in the diseased muscle. However, one might regard the hexose monophosphate, hexose diphosphate and lactate contents as low, in keeping with low glycogenolytic activity.

Discussion.—The case, the clinical features of which will be described in full (Pearson, Rimer and Mommaerts, 1959), is of interest as another example of a metabolic disorder which is referable to the absence of a single enzyme. That the biochemical lesion is specific is shown by the fact that other enzymes associated with phosphorylase are present in normal amounts, particularly phosphorylase kinase and PR enzyme which regulate the level of active phosphorylase. The fact that the accumulated glycogen is normal in structure points to the presence of the branching enzyme. Among the various types of glycogen storage disease, each characterized by the absence of a single enzyme, none has so far been associated with an absence of phosphorylase (G. T. Cori, 1953, 1957). A special feature of the present case seems to be that the enzymatic lesion is apparently confined to skeletal muscle. This is suggested by the fact that there are no cardiac disturbances, that the fasting blood sugar is not abnormally low, and that injection of epinephrine evokes a normal hyperglycemic response.

The observation that the muscles contained 4 per cent glycogen in the virtual absence of phosphorylase makes it clear that there must be another pathway for glycogen synthesis. It has long been suspected that phosphorylase alone cannot account for the synthesis of glycogen since any factor known to activate phosphory-

lase always leads to glycogenolysis rather than synthesis. The original observation by Leloir and Cardini (1957) that there is an enzyme present in liver which can synthesize glycogen from glucose-1-phosphate via uridine diphosphate glucose (UDPG) has now been extended to muscle (Villar-Palasi and Larner, 1958; Robbins, Traut, and Lipmann, 1959; Hauk and Brown, 1959; Hauk, Illingworth, Brown, and Cori, 1959). This synthetic process, unlike that catalyzed by phosphorylase, is not influenced by the concentration of inorganic phosphate and has an equilibrium far to the side of glycogen synthesis. The presence of considerable amounts of glycogen and of the UDPG-glycogen transferase in the muscles of the patient, in the absence of phosphorylase, provides strong support for the operation of the UDPG mechanism for the synthesis of glycogen.

It may be envisaged that normally glycogen is being synthesized at a certain rate by this pathway, but that it is kept at a moderate level by periodic decreases whenever there occurs an increased glycogenolysis associated with exercise, when phosphorylase *b* is activated to phosphorylase *a* (C. F. Cori, 1956; Krebs and Fischer, 1955a; Guillory, Mommaerts, Schottelius and Schottelius, 1959). The elimination of these recurrent decreases would lead to an increased glycogen level, as encountered in the present case. It is of interest, however, that in cases of generalized glycogen storage disease the glycogen content of skeletal muscle may rise as high as 12 per cent, in spite of the fact that phosphorylase is present. The fact that in the present case the glycogen content does not rise to these high values when degradation by phosphorylase seems excluded would indicate that other regulatory factors are in operation which limit the amount of glycogen deposited in muscle.

Summary.—Biopsy specimens of skeletal muscle from a patient with a functional disorder similar to that described by McArdle were analyzed for various enzymes. A homogenate of muscle showed no lactic acid formation from endogenous glycogen, whereas added hexosephosphates were glycolyzed at a normal rate. The enzyme defect was shown to consist in a virtual absence of phosphorylase. Other enzymes related directly or indirectly to glycogen metabolism (phosphoglucomutase, PR enzyme, phosphorylase kinase) were present, and the glycogen had a normal branched structure. The glycogen content (4 per cent) was higher than normal. The presence of the UDPG-glycogen transferase of Leloir and Cardini was demonstrated. Glycogen synthesis in the absence of phosphorylase is ascribed to the action of this enzyme.

The senior author would like to express his indebtedness to Professor C. F. Cori for his considerable interest in the development of this investigation, and for the cooperation of his department in several parts of the experimental work.

* Dedicated to the memory of Gerty T. Cori in recognition of her pioneer work on the biochemistry and pathology of glycogen storage diseases.

† With support of Grant No. H-3067 from the National Heart Institute of the National Institutes of Health, Bethesda, Md., and of a grant from the Muscular Dystrophy Associations of America, Inc.

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*ACTION SPECTRA FOR THE POSITIVE AND NEGATIVE PHOTOTROPISM OF PHYCOMYCES SPORANGIOPHORES**

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Communicated by Kenneth V. Thimann, April 20, 1959

One of the most effective approaches to the study of the mechanism of phototropic perception is by means of the action spectrum. In the case of the phototropism of *Phycomyces* sporangiophores in visible light, the action spectrum has been studied by Blaauw,[2] Castle,[5] Wiechulla,[18] and Bünning,[6] and an analysis of Wiechulla's results was published by Buder.[3] These investigators obtained single-peaked action spectra with a maximum phototropic effectiveness in the blue region, which, according to Wiechulla, is also the most effective region in promoting straight growth. Working with *Pilobolus*, a genus related to *Phycomyces*, Bünning found two maxima of phototropic effectiveness in the blue for sporangiophores with subsporangial vesicles,[4] and only one maximum for those without these vesicles.[6] However, the results obtained by these earlier workers are not sufficient to clarify the details of the action spectra because filters with broad transmission bands were

used. Furthermore, calculations of the energy relationships were insufficiently precise.

Recently Curry and Gruen[10] described the negative phototropism and growth promotion of *Phycomyces* sporangiophores in the ultraviolet, and gave preliminary data for the curvature response at several wave lengths. The response to a few wave lengths in the visible range was also included. The following report will present the details of the action spectra for phototropic curvature of this fungus in visible and in ultraviolet light.

Materials and Methods.—In these experiments the plants were exposed to continuous light of graded intensities, the method used being a balancing system, whereby the plant is used as a photometer. It is essentially the same method as that first used by Massart[14] for *Phycomyces* and was selected because it is very suitable for use with continuous exposures. The plants were three- or four-day old cultures of *Phycomyces blakesleeanus* (G-5, + strain) grown on potato-dextrose agar in 10 or 25 ml beakers exposed to an overhead red-fluorescent lamp wrapped with several layers of red cellophane. This lamp caused no observable light-growth reactions when sporangiophores were exposed to it after adaptation in total darkness for periods of thirty minutes or longer. It was assumed, therefore, that plants grown under this lamp were completely "dark-adapted." For the balancing experiments the beakers were placed at intervals on a 2 meter long plank. One end of this plank was fixed beneath the exit lens of a Bausch and Lomb grating monochromator with quartz optics (dispersion: 3.3 mμ/mm) and the other end beneath a 6-volt lamp mounted in a cylinder with two 4-mm Corning filters (No. 5031 and No. 4303) over the aperture. The latter constituted the "standard" blue light (spectral center *ca.* 460 mμ) against which the various monochromatic wave lengths were balanced. The monochromator source was either a GE H85C3 mercury lamp or a GE ribbon-filament tungsten lamp.

Cultures were exposed simultaneously to the two oppositely directed light beams, impinging on the sporangiophores at an angle slightly above the horizontal. After one or two hours of continuous exposure the plants were examined; usually, a single culture could be found in which sporangiophores were bent in both directions. On either side of this culture were beakers with sporangiophores bent predominantly (90–100%) either toward the monochromator or toward the standard blue light. In cases where the balance point was not clear, the cultures were rotated 90° and the balance redetermined. Finally, a few cultures were placed close together near the balance point and a final balance position obtained in the next hour or so of exposure. This procedure was repeated for a series of wave-length settings of the monochromator throughout the visible and near ultraviolet spectrum.

When using the mercury lamp, the relative quantum intensities of the monochromator output ($Q_{M\lambda}$) and the standard blue light (Q_B) at balance were obtained using a photocell whose spectral sensitivity had been determined by calibration with an Eppley thermopile. The absolute intensities at the middle of the plank were less than 5 ergs/cm^2 sec. The ratio of the relative quantum intensities at balance ($Q_B/Q_{M\lambda}$) was taken as a measure of the relative effectiveness of each spectral region. At 436 mμ the relative effectiveness value was arbitrarily set at 1.00 to facilitate the comparison of the data obtained from experiments with the mercury and tungsten lamps.

In a long series of experiments with the tungsten lamp the relative effectiveness values were determined by a somewhat different procedure, mostly because the absolute monochromatic intensities at the middle of the plank were very much lower in this case (<0.5 ergs/cm² sec). First, the monochromator was set for transmission of 530 mμ and the intensity was measured as a function of the distance from the monochromator exit, using an amplified photomultiplier as a detector. Next the intensity of the standard blue light as a function of distance along the plank was measured by the same method. Finally, the relative energy output at each monochromatic wave length was determined by taking readings from the thermopile connected to a Kin-Tel D.C. amplifier, the thermopile being at a fixed distance from the monochromator exit lens. Putting these three series of observations together permitted the calculation of the relative quantum intensity ratios ($Q_B/Q_{M\lambda}$) as a function of position on the plank and wave length of the monochromatic light. Then for each balance point the relative effectiveness values were found by reference to these data, again setting 436 mμ at 1.00. Since this method depended on a single series of energy determinations, it was subject to errors due to fluctuations in the light sources over long periods of time. However, the particular balance points were quite repeatable, indicating that the light sources were stable and the plants unchanging in their spectral sensitivity from one day to another.

Results.—In practice it was found that balance points could easily be obtained within ±2.5 cm. The positions were remarkably consistent considering the relative simplicity of the method. In fact, the same positions were found whether the plants were completely or incompletely dark-adapted beforehand. *Phycomyces* sporangiophores seem to be excellent "plant photometers" in Johnston's sense.[13]

TABLE 1

RESULTS OF A SERIES OF EXPERIMENTS IN WHICH MONOCHROMATIC LIGHT FROM A TUNGSTEN SOURCE WAS BALANCED AGAINST THE "STANDARD BLUE" LIGHT

Wavelength λ mμ	Balance Points Observed (Cm from Monochromator)	Avg. Balance Position	Number of Determinations	Relative Effectiveness: $Q_B/Q_{M\lambda}$ at Bal.
350	≤20	≤20	1	≤ .40
380	52, 48, 45, 45	47.5	4	.83
390	40	40	1	.62
400	57.5, 62, 61, 61	60.4	4	.74
410	62.5, 55	58.8	2	.55
415	60, 62.5	61.3	2	.59
420	70	70	1	.86
425	65, 77	71	2	.82
430	75, 77.5	76.3	2	1.00
435	75, 80	77.5	2	.98
440	92.5, 90, 87.5, 86.5	89.1	4	1.76
445	90, 90, 92.5, 92.5, 92.5	91.5	5	1.79
450	92.5, 92.5, 92, 93.5, 93.5	92.8	5	1.82
455	92.5, 87.5	90	2	1.46
460	90, 90, 93, 94, 92.5, 92.5, 89, 93, 92.5, 92.5, 93, 95, 95, 95	92.6	14	1.52
465	94.5	94.5	1	1.54
470	97.5, 99, 96, 95, 97.5, 96	96.8	6	1.57
475	97.5, 95, 95, 93, 95, 95, 95, 95	95.1	8	1.36
480	100, 95, 91.5	95.5	3	1.33
485	85, 85	85	2	.75
490	97.5, 85, 75	85.8	3	.74
495	77.5	77.5	1	.45
500	67.5, 70, 70	69.2	3	.28
505	<52.5	<52.5.	1	< .10
525	<20	<20	1	< .02

The direct results of the experiments with the tungsten lamp are tabulated below (Table 1).

The data on balance positions lead directly to a rough action spectrum, even without reference to the intensity measurements. With a tungsten source the output of the monochromator with fixed slit widths increases steadily as the wave length is changed from 350 to 550 mμ, the curve being very similar to the usual tungsten emission curve. With this in mind it may be noted that the balance position shifts more or less steadily away from the monochromator as the wave length is increased. Beyond 480 mμ, however, even though the intensity at each wave length is still increasing, the balance positions now shift back toward the monochromator. Clearly, a maximum effectiveness is indicated in the region between 430 and 480 mμ. The fact that the balance positions do not change as

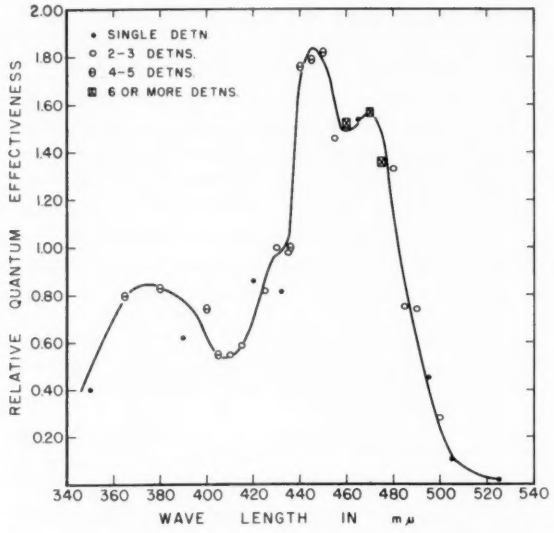

FIG. 1.—The action spectrum for the positive phototropism of *Phycomyces* sporangiophores.

smoothly as the intensities in this range also implies that there is not a single broad maximum.

Below 400 mμ the low output from the tungsten source introduces the possibility of relatively large errors. For this reason several determinations were made with a mercury lamp to check the effectiveness of 365 and 405 mμ, relative to that of 436 mμ, taken as 1.00. At least 5 balances were determined at each of these wave lengths, using Corning glass filters in conjunction with the monochromator to ensure spectral purity. The values from these experiments together with the relative effectiveness values from Table 1 have been plotted in Figure 1 to give the action spectrum. This spectrum shows a maximum of effectiveness at 445 mμ, a shoulder at 470 mμ, and a secondary maximum near 370 mμ. The fall-off on the long wave length side is quite abrupt, activity being negligible above 525 mμ.

On the short wave-length side, light throughout the near ultraviolet range continues to be phototropically effective.

An attempt was made to see how the spectral sensitivity of the Light Growth Reaction (growth acceleration following symmetrical illumination) compared to this action spectrum for phototropism. Modifying the methods used by Delbrück and Reichardt,[11] measurements were first attempted on individual sporangiophores, but the variability was so large that one could only conclude that blue light is most effective. Subsequently, an arrangement was made whereby a whole culture could be shadowgraphed at three time intervals, using inactive red light. The culture was placed in a lucite box covered on top by a diffusing plate. The monochromator output was directed on to the surface of this plate so that the plants could be given a diffuse stimulus from above. After a 20 to 30 minute period of adaptation to the red working light, the first shadowgraph was taken. Four minutes later the monochromatic stimulus was given, and after two more minutes (which is a little less than the latent period) a second shadowgraph was superimposed on the first. After 6 more minutes a third shadowgraph was superimposed. Thus the difference between the first and the second image measures the growth during 6 minutes before the Light Growth Reaction, and the difference between the second and third image gives the growth in 6 minutes during the reaction. The ratio of the latter to the former, measured for at least 20 sporangiophores, was taken as a measure of the response. In Figure 2 are plotted the growth promotion ratios for 1, 4, 16, and 64 second exposures to low intensity 460 mμ light. It is apparent that the response is a log-linear function of the dose, as described by Delbrück and Reichardt.[11] Unfortunately, however, small changes in dose lead to very small absolute changes in the ratios measured; hence, small changes in spectral effectiveness could easily be missed. The comparison of results at different wave lengths is further obscured by variations presumably produced by small changes in temperature, lighting, or in the age of the cultures. Although not permitting the construction of a detailed action spectrum, the results of these studies indicated a broad maximum of effectiveness, possibly two-peaked, in the region between 430 and 490 mμ. The fall-off on the long wave-length side is abrupt, the relative effectiveness of 520 mμ being less than 2 per cent that of 460 mμ. The fall-off on the short wave-length side is much less abrupt, and 365 mμ remains relatively effective. All in all, then, the action spectrum for the Light Growth Reaction is very similar to that for phototropism, and as has already been suggested[18] they may well be identical.

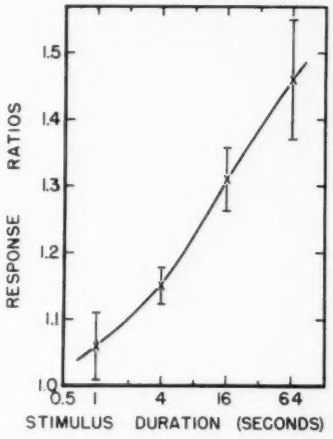

FIG. 2.—The promotion of growth by light expressed as the ratio of growth during the first 6 minutes of the Light Growth Reaction to the growth in the preceding 6 minutes, plotted as a function of the length of exposure to constant low intensity light of 400 mμ. Each point is the mean of 20 observations. The vertical bars indicate ± twice the standard error of the mean.

The spectral sensitivity of the negative curvatures caused by exposure to ultraviolet light of wave lengths shorter than 312 mμ[10] was also investigated using the balancing method. In this case a small germicidal lamp (Westinghouse Sterilamp, WL-793, 4 watts) shining through a Corning 9863 filter was used instead of the "standard blue" light; i.e., the filtered sterilamp was the "standard UV" source, its output being almost exclusively light of wave length 254 mμ. The same 2-meter plank was used and light intensities measured with a calibrated photocell (GE 8PV1,DAB, high UV sensitive). The opposing source was the monochromator with the H85C3 mercury lamp. In these experiments balances could be found much more rapidly than in the work with visible light because the rate of curving in ultraviolet is faster, as noted previously.[10] Usually 20 or 30 minutes sufficed for a balance determination, each being repeated several times. The accumulated results

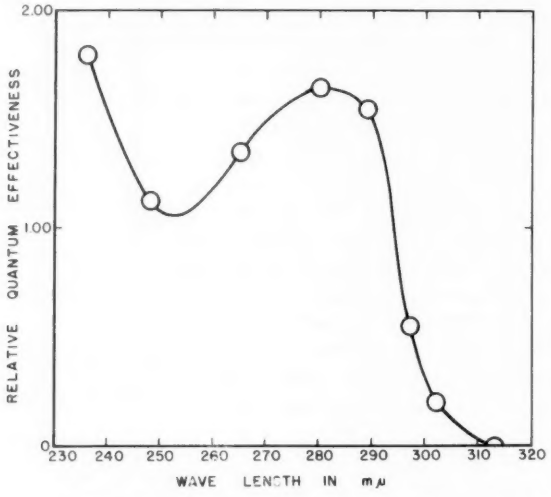

FIG. 3.—The action spectrum for the negative phototropism of *Phycomyces* sporangiophores.

are shown as an action spectrum in Figure 3. This spectrum is not as detailed as that in the visible because fewer useful wave lengths are available from the monochromator in the UV region, and the intensity measurements are not as precise. The curve shows a single peak near 280 mμ, a trough near 250 mμ, and a steady rise below this. Above 290 mμ the curve falls abruptly, the negative effect vanishing near 312 mμ which elicits little or no curvature, either positive or negative. Positive responses are again found at 334 mμ.

As described previously,[10] the negative curvatures are associated with a positive Light Growth Reaction; presumably they result from an acceleration of growth in the front side of the sporangiophore. Precise measurements of Light Growth Reactions caused by light of wave lengths shorter than 312 mμ are difficult, but symmetrical illuminations with 302, 312.5, and 334 mμ were all observed to give positive Light Growth Reactions of approximately the same magnitude. Under

the same circumstances but given unilaterally, 302 mμ causes negative curvatures, 312.5 mμ causes no curvature, and 334 mμ gives positive curvatures (cf. Figs. 1 and 3). Therefore, it seems reasonable to interpret the "null point" of curvature response near 312 mμ as a point where Light Growth Reactions in the front and back sides of the sporangiophore are balanced.[1,7]

Discussion.—In the blue spectral region the action spectrum is remarkably similar to the absorption spectrum of carotenoid pigments known to be present in *Phycomyces*,[5, 12] although the secondary maximum near 370 mμ is not characteristic of these carotenoids. The absorption spectrum of riboflavin does not show two peaks in the blue region and has a much higher peak near 365 mμ; hence the action spectrum does not support the idea, suggested by Reinert[15] and by Carlile[7], that riboflavin is the photoreceptor. It is interesting to point out that the visible action spectrum for *Phycomyces* is almost identical with that of *Avena* coleoptiles as determined by Shropshire and Withrow,[16] and by Curry,[9] and Thimann and Curry;[17] the maxima at 445, 470, and 370 mμ may be compared with 445, 474, and 370 in *Avena*.[17] Thus the photoreceptors in the two plants are identical or very closely related.

The evidence strongly suggests that the Light Growth Reaction and phototropism in *Phycomyces* are initiated by the same light reaction.[2] However, the evidence is insufficient at the moment to establish the relationship between the ultraviolet and visible light mechanisms.

Summary.—The action spectra for phototropism of *Phycomyces* were obtained by using the curvature of the sporangiophores to balance a standard blue or ultraviolet light against narrow band emission from a monochromator. In the visible (positive curvature) the spectrum shows a main peak at 445 mμ with a shoulder at 470 mμ and a secondary peak at 370 mμ. In the ultraviolet (negative curvature) there is a single peak near 280 mμ. In both cases growth promotion accompanies the curvatures, and in the visible range the Light Growth Reaction shows probably the same spectral sensitivity as does the curvature; it is suggested that curvature and growth promotion are initiated by the same reaction. The photoreceptor of *Phycomyces* in the visible is very similar to, or may be identical with, that of *Avena* coleoptiles. Both are probably carotenoids.

The authors are grateful to Professor K. V. Thimann for his encouragement and valuable suggestions in the course of this work, and for critically reading the manuscript. The authors also wish to thank Mrs. Irmgard Kurland for her help in drawing the illustrations.

* Supported in part by a grant from the National Science Foundation to Professors K. V. Thimann and R. H. Wetmore.

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THE FUOSS-ONSAGER CONDUCTANCE EQUATION AT HIGH CONCENTRATIONS

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Communicated April 24, 1959

The problem of accounting for the decreasing equivalent conductance of electrolytic solutions with increasing concentration has engaged the attention of physical chemists for almost a century. A partial solution of this problem was provided by the theory of Onsager.[1] While this theory shed much light on some phases of the conductance problem, it has failed to reproduce observed conductance values quantitatively even at rather low concentrations. The reason for this lies in the fact that, in the development of the theory, the ions were treated as point charges. Moreover, terms of order higher than $C^{1/2}$ were not evaluated.

In a recent paper, Fuoss and Onsager[2, 3] have obtained a solution for the relaxation and electrophoretic terms for charged spheres and they have evaluated significant terms of order higher than $C^{1/2}$. Their conductance equation may be written:

$$\Lambda = \Lambda_0 - SC^{1/2} + JC + EC \log C$$

The form of this equation confirms Onsager's earlier theoretical predictions[1, 4] and Shedlovsky and Brown's[5] experimental results. The coefficient, S, of the $C^{1/2}$ term is the limiting Onsager slope. The coefficients J and E involve the absolute temperature, the dielectric constant and viscosity of the solvent medium and the number of unit changes on each ion. In addition, they involve the parameter, Λ_0, the limiting conductance, and J involves the parameter a, the distance of closest approach of the centers of ions in contact.

Fuoss and Onsager[2] have shown that their equation reproduces observed conductances for unassociated 1-1 electrolytes in water within the limit of experimental

error up to a concentration where κa begins to have a value greater than 0.2. Above this limiting concentration, the theory is not applicable as the authors are careful to point out.

Shortly before the advent of the Fuoss-Onsager theory, one of us (Accascina) had measured the conductance of tetramethylammonium picrate in a 55 per cent dioxane-water mixture from low concentrations to 0.227 N, near the limit of solubility of the electrolyte. Somewhat earlier, Mercier and Kraus[6] had measured the conductance of the same electrolyte up to 3×10^{-3} N in a series of mixtures from water to 70 per cent dioxane. Fuoss and Kraus[7] analyzed the data of Mercier and Kraus on the basis of the Fuoss-Onsager theory. They found that the observed conductances were reproduced by the theory using only the two parameters Λ_0 and a. This indicated that the electrolyte was unassociated in most of the systems and only slightly associated, if at all, in the solvent mixtures where the dielectric constant was as low as 19.

In view of this result, there could be little doubt that, in a 55 per cent mixture of dielectric constant 31.5, the electrolyte is completely dissociated. The data which follow, therefore, afford a means for testing the applicability of the Fuoss-Onsager theory to solutions in a solvent having a dielectric constant much lower than that of water.

In Table 1 the values of the concentration and conductance are given in columns 1 and 2, respectively. The data of Mercier and Kraus are included and are designated by an asterisk.

TABLE 1

CONDUCTANCE OF Me_4NPi IN A 55% DIOXANE–WATER MIXTURE

10^3C	Λ	$\Delta\Lambda$	10^3C	Λ	$\Delta\Lambda$
1.85315	32.333*	−0.012	517.500	24.637	− 15.5
3.70072	31.951*	−0.024	724.447	23.735	− 25.2
7.35544	31.434*	−0.005	966.954	23.130	− 37.2
8.13515	31.343	−0.005	1126.09	22.569	− 45.5
9.39140	31.216	+0.004	1141.83	22.625	− 46.5
14.4515	30.795	+0.027	1457.06	21.724	− 64.4
14.7007	30.776*	+0.026	1516.84	21.599	− 67.5
29.3854	29.943*	−0.005	1822.74	20.797	− 86.2
49.7268	29.315	−0.053	1831.62	20.793	− 86.7
97.4673	28.374	−0.648	2059.86	20.373	−100.7
132.8561	27.571	−1.68	2259.90	19.894	−113.4
387.846	25.567	−9.92	2265.82	19.840	−113.9

Viscosities are shown in Table 2. The viscosity for the solvent is that of Geddes[8] as corrected by Mercier.[9]

TABLE 2

VISCOSITY OF SOLUTIONS OF Me_4NPi IN A 55% DIOXANE–WATER MIXTURE

C	0	0.0535	0.1003	0.2055
$10^2\eta$	1.964	2.028	2.094	2.193

Using a least squares treatment, values of the limiting conductance, Λ_0 and the ion size parameter, a, were determined from the conductance values at concentrations less than 3×10^{-3} N. The values obtained were $\Lambda_0 = 37.39$ and $a = 6.14$ Å. With these values of the parameters, the normal conductance equation becomes:

$$\Lambda = 37.39 - 73.34\, C^{1/2} + 755.6C + 214.8C \log C$$

On the basis of this equation, conductances were computed for all concentrations. The calculated values were subtracted from the observed values yielding the difference, $\Delta\Lambda$, between the two. Values of $\Delta\Lambda$ are shown in columns 3 and 6 of Table 1.

Inspection of these values shows that, up to about 3×10^{-3} N, the equation reproduces the observed values within the limit of experimental error. For 8 concentrations, the average difference is 0.014 A-unit or 0.05 per cent. At concentrations above 3×10^{-3} N, the calculated values begin to diverge from the observed and the differences became very large at high concentrations. At the highest concentration, $C = 0.227$ N, the calculated conductance is 6.7 times that observed.

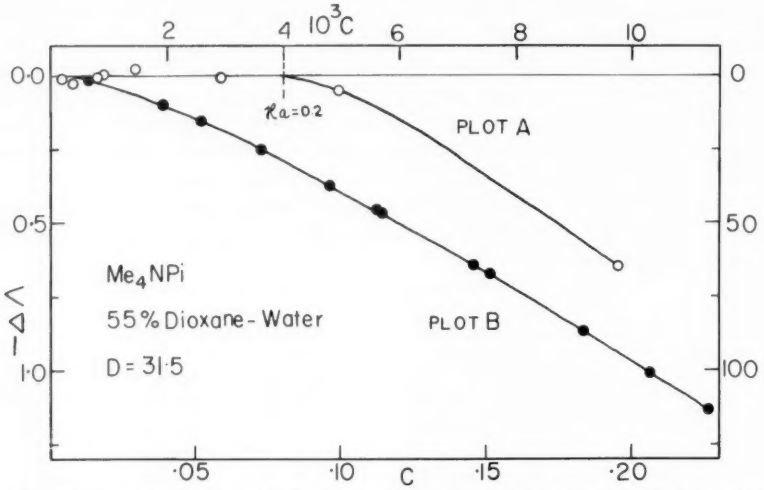

FIG. 1.—Deviation between observed and calculated equivalent conductances: Plot A, dilute range, coordinates left and above; Plot B, entire range, coordinates right and below.

The manner in which calculated and observed values diverge is shown graphically in Figure 1. In Plot A, the scale of ordinates is such that differences may be seen at the lowest concentration. Two points are not included since they are practically coincident with the plotted points. It can be seen that these differences, as already noted, are random and within the limit of experimental error. Above 3×10^{-3} N, the differences increase in a systematic fashion.

Fuoss and Onsager state that their theory is applicable up to concentrations where κa is less than about 0.2. For Me_4NPi in water, the limiting concentration is 0.01 N, while in a 55 per cent dioxane-water mixture, it is 0.004 N. This predicted value is in good agreement with our observations as exemplified by plot A, Figure 1.

In plot B, the entire concentration range is covered up to 0.227 N. At low concentrations, differences are indiscernible on the scale of the plot. Above about 0.01 N, they become appreciable and become very large at high concentrations. At the highest concentration, $\Delta\Lambda = -113.9$.

The large differences at higher concentrations can, obviously, not be due to any slight error in the coefficients or in the underlying theory. As Fuoss and Onsager pointed out, the theory does not apply at concentrations above the limit which they have set on the basis of the theory itself.

Several important conclusions may be drawn from the results of the present investigation:

1. The theory of Fuoss and Onsager is applicable to solvents of dielectric constant much lower than that of water.

2. In solvents of lower dielectric constant, as in water, the Fuoss-Onsager theory is applicable to solutions for which the value of κa is not greater than 0.2.

3. It may be anticipated that any attempt to account for the conductance of electrolytic solutions at high concentrations on the basis of the current ion-atmosphere model will prove futile.

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*DEPENDENCE OF THE WALDEN PRODUCT ON DIELECTRIC CONSTANT**

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Read before the Academy, April 27, 1959

If the ions of an electrolytic solution are represented by spheres of radius R and solvent is assumed to be a continuum described by its macroscopic viscosity η_0, then Stokes hydrodynamics leads directly to the velocity

$$v_0 = Xe/6\ \pi\eta_0 R \tag{1}$$

for an ion of charge e moving in a field of strength X esu/cm. In terms of the limiting equivalent conductance λ^+_0 for a cation, this relation becomes

$$\lambda^+_0 = \mathfrak{F}e/1{,}800\ \pi\eta_0 R^+ \tag{2}$$

where $\mathfrak{F}$ is the Faraday equivalent. The sum of (2) and the corresponding expression for the anion gives

$$\Lambda_0\eta_0 = (\mathfrak{F}e/1{,}800\ \pi)(1/R^+ + 1/R^-) \tag{3}$$

The right side of (3) should be constant for a given electrolyte, and therefore (3) can be written as Walden's Rule:

$$\Lambda_0\eta_0 = \text{const.} \tag{4}$$

It is a matter of experience, however, that (4) is at best an approximation: the product of limiting conductance times solvent viscosity varies by about a factor of two on going from water to organic solvents, and hydrodynamic radii calculated by (2) from single ion conductances frequently give unrealistic values which show little correlation with the structure of the electrolyte, and which vary widely from solvent to solvent for a given electrolyte. The concept of "solvation" has frequently been invoked in an attempt to rationalize these discrepancies. It is the purpose of this paper to suggest a different origin for the inconstancy of the Walden product: electrostatic interaction between the fields of the moving ions and those of the solvent dipoles in the surrounding solvent.

In order to formulate the problem, let us consider several typical examples, shown in Figure 1 which represents the dependence of viscosity (*a*) and of the Walden product (*b*) for tetrabutylammonium bromide on solvent composition for solvent systems water-dioxane[1] (I), nitrobenzene-carbon tetrachloride[2] (II) and methanol-carbon tetrachloride[3] (III). The abscissa scale is weight per cent w of the nonpolar component of the mixture. In I, viscosity goes through a maximum as w increases; in II, viscosity decreases with increasing w, while in III, viscosity increases as w increases. There is no correlation whatsoever between the dependence of $\Lambda_0\eta_0$ on composition and the dependence of viscosity on composition; we therefore conclude that the inconstancy of $\Lambda_0\eta_0$ must be controlled of some other variable of the system than viscosity. It will, however, be noted that the three *b*-curves have one characteristic in common: $\Lambda_0\eta_0$ decreases as w increases, that is, as the dielectric constant of the solvent decreases. We are thus led to consider the effect of electrostatic forces in the system on the hydrodynamics.

Around a "stationary" ion (i.e., ignoring its Brownian motion), the nearby solvent dipoles will assume a spherically symmetric distribution of orientations with the reference ion at the center; "nearby" dipoles, because ion-dipole potential energy decreases as the inverse square of the distance between ion and dipole. If the charge on the reference ion were suddenly annihilated, the dipoles would revert to the distribution demanded by their mutual fields, a distribution which would be essentially random with respect to the origin at the discharged ion. But the relaxation of the orientation would not occur instantaneously, because the solvent has a nonzero viscosity, and it therefore requires a finite time for dipole rotation to occur. Consequently, if we consider an ion moving through a solvent, we would expect it to set up a steady state in the distribution of orientations of neighboring solvent molecules which would lag behind the equilibrium distribution around the ion at rest. Too few dipoles would be oriented toward the ion in the direction of its motion, while behind it, too many dipoles would still be pointing in its general direction. The net effect therefore is that the motion of the ion creates an electrostatic field in the surrounding polar medium which opposes its motion. Alternatively stated, the electrostatic coupling between the ion and the solvent acts effectively as an increase in viscosity, in that extra work must be done to orient the dipoles as the ion passes among them. The local viscosity then would be of the form

$$\eta = \eta_0 + \Delta\eta \tag{5}$$

The viscosity increment should be proportional to η_0, because the rotatory friction coefficient[4] of a solvent molecule varies as η_0. It should be proportional to the moment of the solvent molecules, and assuming continuum theory, inversely proportional to the dielectric constant of the solvent. Since the extra work would involve an integral from the surface of the ion to "infinity," we might expect $\Delta\eta$ to vary as R_∞^{-2}, where R_∞ is the hydrodynamic radius of the ion in a hypothetical solvent of infinite dielectric constant where all electrostatic forces would be reduced to zero. If these heuristic arguments are combined, (5) becomes

$$\eta = \eta_0(1 + A/DR_\infty^2) + O(D^{-2}) \tag{6}$$

and then Stokes equation becomes (in conductance units)

$$\lambda_0\eta_0 = \mathfrak{F}e/1{,}800\ \pi R_\infty(1 + A/DR_\infty^2) \tag{7}$$

If we designate by R the classical Stokes radius, as calculated by Walden,

$$R = \mathfrak{F}e/1{,}800\ \pi\lambda_0\eta_0 \tag{8}$$

and then (7) and (8) give

$$R = R_\infty + B/D + O(D^{-2}) \tag{9}$$

where R is an experimentally determinable quantity, given single ion conductances.

Transference numbers are, of course, known for many ions in aqueous solution, but are relatively scarce for nonaqueous solutions. In order to test Equation (9) experimentally on a variety of systems, it will therefore be necessary to make several assumptions. The following analysis will be based on two assumptions: (1) The limiting transference number is independent of solvent composition in a mixture of a polar and a nonpolar solvent. (2) The limiting transference numbers of both ions of tetrabutylammonium tetraphenylboride are equal to one half in all solvents.

Mercier and Kraus have reported[1] the conductance of tetrabutylammonium bromide in water-dioxane mixtures, covering the range $78.5 \geqslant D \geqslant 19.07$ (0–70 per cent dioxane); limiting conductances have been calculated[5] by the method of Fuoss[6] and are summarized in Table 1. Using[7] $\lambda_0(\mathrm{Br}') = 78.20$ for aqueous solutions, the limiting transference number of the bromide ion is 0.8020. Using assumption (1), the single ion Walden products $\lambda_0^+\eta_0$ amd $\lambda_0^-\eta_0$ were calculated, and from these, by means of equation (8), the Stokes radii R^+ and R^-. A plot‡ of RD against D for the tetrabutylammonium ion (Line I) and for the bromide ion (Line II) is shown in Figure 2. The points can be approximated very closely by a straight line; the method of least squares gives for Line I

$$R^+D = 21.484 + 4.461D \tag{10}$$

Values of R^+ calculated by equation (10) are given in Table 1, and deviations between calculated and observed values are shown in the last column: $R_\infty = 4.461 \pm 0.049$. While the deviations are systematic, they are small (max, 1.6 per cent; standard deviation 1.0 per cent). The very near linearity of Lines I and II of Figure 1, as contrasted with the strong curvature of I *b* of Figure 1, is encouraging support for the arguments which led to equation (9).

We next consider a variety of salts in nitrobenzene-carbon tetrachloride mixtures.

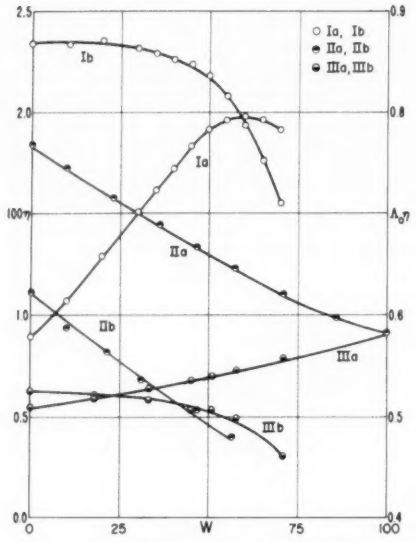

FIG. 1.—Dependence of viscosity (*a*) and Walden product (*b*) on composition for tetrabutylammonium bromide in water-dioxane (I), nitrobenzene-carbon tetrachloride (II), and methanol-carbon tetrachloride (III) mixtures.

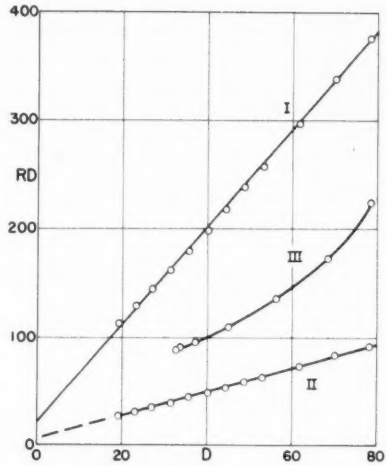

FIG. 2.—Test of equation (9) for Bu_4N^+ in water-dioxane mixtures (I), for Br^- in water-dioxane (II), and for Et_4N^+ in water-methanol (III).

The top line of Figure 3 is based on data for tetrabutylammonium tetraphenylboride;[8, 9] as seen, the points lie on a straight line. By interpolating at the corresponding points on a plot of $(\Lambda_0\eta_0/2)$ against D for this salt, values of $\lambda^+_0\eta_0$ are obtained for solvent mixtures of compositions corresponding to those in which other tetrabutylammonium salts were measured; then $(\Lambda_0\eta_0 - \lambda^+_0\eta_0)$ gives the

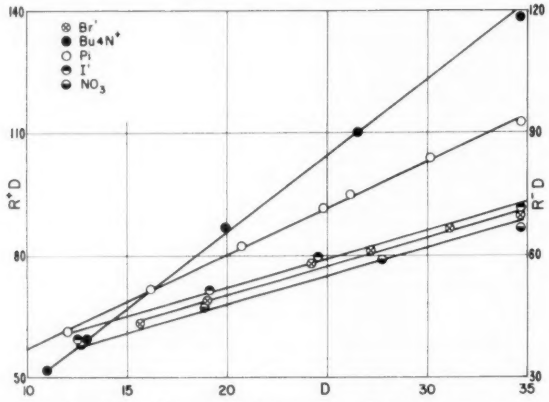

FIG. 3.—Dependence of Stokes radii on dielectric constant for $Bu_4N^+ = BPh_4^-$, Pi^-, I^-, Br^-, and NO_3^- ions in nitrobenzene-carbon tetrachloride mixtures.

anionic term. The *RD-D* plots for the picrate,[9] iodide,[9] bromide,[2] and nitrate[9] are also shown in Figure 3; they are likewise linear. The slopes of the lines are proportional to R_∞; it will be noted that the slopes in Figure 3 are in the sequence expected from the sizes of the sequence of ions: $Bu_4 = BPh_4^- > Pi^- > I^- \approx Br^- \approx NO_3^-$.

TABLE 1

TETRABUTYLAMMONIUM BROMIDE IN DIOXANE-WATER MIXTURES

$C_4H_8O_2$, %	Λ_0	$10^6 R^-$ (obs.)	$10^6 R^+$ (obs.)	$10^6 R^+$ (calc.)	$10^6 \Delta R$
0	97.50	1.176	4.795	4.735	−0.060
10	80.80	1.176	4.800	4.766	−0.034
20	67.59	1.173	4.783	4.808	0.025
30	57.29	1.183	4.826	4.864	.038
35	53.10	1.190	4.857	4.900	.043
40	49.45	1.198	4.883	4.943	.060
45	46.30	1.204	4.912	4.995	.083
50	43.71	1.220	4.978	5.060	.082
55	41.57	1.250	5.099	5.142	.043
60	39.76	1.296	5.286	5.251	−0.035
65	38.31	1.358	5.536	5.389	−0.053
70	37.13	1.436	5.857	5.588	−0.031

In Figure 4 are shown similar plots for tetrabutylammonium ion (or tetraphenylboride ion) in acetonitrile-carbontetrachloride[10] mixtures, for the two ions of tetrabutylammonium bromide in methanol-carbon tetrachloride[3] mixtures, and for the latter salt in ethanol-carbon tetrachloride[11] mixtures. (Gordon's value[12] $n^- = 0.5871$ was used to evaluate λ_0^- in methanol, and the same value of n^- was assumed for ethanol.) The first three give excellent straight lines; the points for the ethanol systems scatter somewhat, but are linear within the experimental uncertainty (which was a consequence of the photochemical sensitivity of the C_2H_5OH-CCl_4 system).

Finally, in Figure 4 are also shown a set of points for tetrabutylammonium bromide in methanol-nitrobenzene[13] mixtures. Here we have a mixture of two polar solvents: up to about 90% nitrobenzene, the points lie close to the MeOH-CCl_4 lines; in the nitrobenzene-rich systems, a sharp deviation appears. This may mean selective

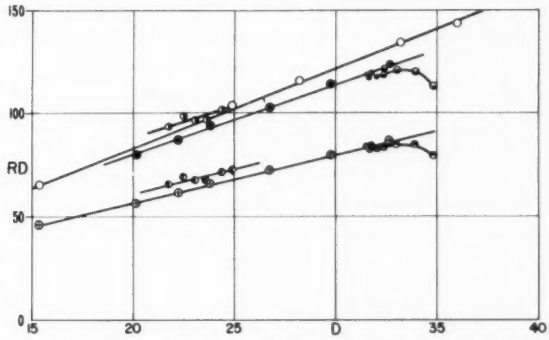

FIG. 4.—Test of equation (9) for: ◑, Bu_4N^+ in EtOH-CCl_4; ○, $Bu_4N^+ = BPh_4^-$ in MeCN–CCl_4; ●, Bu_4N^+ in MeOH-CCl_4; ◒, Bu_4N^+ in MeOH-$PhNO_2$; ◐, Br^- in EtOH-CCl_4; ⊕, Br^- in MeOH-CCl_4 and ◓, Br^- in MeOH-$PhNO_2$.

solvation by methanol, but other rationalizations are possible. Another example of a polar-polar mixture is shown as Curve III of Figure 2, for tetraethylammonium picrate in methanol-water mixtures.[14] Again a nonlinear plot is obtained. Evidently when two polar solvents are present, the situation is complicated by specific competitive effects.

TABLE II

SUMMARY OF DERIVED CONSTANTS

Ion	Solvent	$10^8 R_\infty$	B
Bu_4N^+	*H_2O-$C_4H_8O_2$	4.46	21.5
Bu_4N^+	*MeOH-CCl_4	3.35	14.0
Bu_4N^+	*EtOH-CCl_4	3.53	17.0
Bu_4N^+	$PhNO_2$-CCl_4	3.55	15.0
Bu_4N^+	MeCN-CCl_4	3.70	10.5
Br^-	*H_2O-$C_4H_8O_2$	1.09	6.0
Br^-	*MeOH-CCl_4	2.40	8.0
Br^-	*EtOH-CCl_4	2.60	9.0
Br^-	$PhNO_2$-CCl_4	1.34	23.5
I^-	$PhNO_2$-CCl_4	1.61	20.5
NO_3^-	$PhNO_2$-CCl_4	1.37	19.5
Pic^-	$PhNO_2$-CCl_4	2.36	13.0

A summary of the constants for the various systems is given in Table 2. In Figure 5A, the values of B are plotted against R_∞; aside from the obvious fact that the points lie on a plane, no correlation appears at first glance. Further scrutiny, however, reveals a surprising correlation. On Figure 5B are plotted the values for the systems marked with an asterisk in Table 2; it will be noted that for those systems, B increases as R_∞ increases. In Figure 5C are plotted the values for the remaining systems; here B decreases as R_∞ increases. The latter behavior conforms to the guess underlying equation (6). The solid curve in Figure 5C is the rectangular hyperbola $R_\infty B = 36$, where 36 is the average of the $R_\infty B$ products for the six systems of the figure. The segregation made in Figures 5B and 5C parallels a characteristic of the solvent system: the solvents in 5B all contain hydrogen-bonding components, while those for 5C are aprotonic. This correlation may be fortuitous, although the strong departure of Curve 2 III from linearity (H_2OMeOH) argues that hydrogen bonding also plays a role in the local hydrodynamics near an ion.

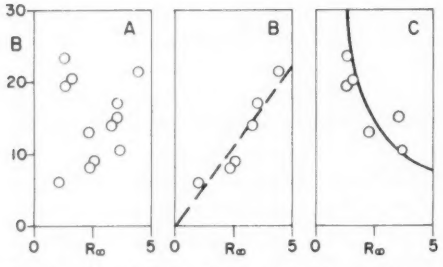

FIG. 5.—Correlations of constants of equation 9.

The magnitudes of the limiting hydrodynamic radii are quite reasonable, and parallel what might be expected from the structure of the ions. Fair agreement (3.35–3.70) is found for Bu_4N^+ in four quite different solvent systems. In the $PhNO_2$-CCl_4 system, the sequence parallels the model sizes for the tetraphenyl boride, picrate, iodide, bromide, and nitrate ions. The calculated size of the bromide ion depends on the nature of the solvent; when MeOH is present, the effective hydrodynamic entity which corresponds to Br^- is about twice as large as

in the aprotonic mixture $PhNO_2$-CCl_4. There are, of course, too few data available at present to justify much speculation; on the other hand, the results summarized in Table 2 and Figure 5B and 5C suggest that an investigation of a wide variety of salts and solvent mixtures would lead to useful information.

If the model used in the calculation corresponds to the actual physical system, then the sum $\Sigma = (R^+{}_\infty + R^-{}_\infty)$ should equal the value obtained for a, the center-to-center distance of anion and cation at contact. This parameter can be calculated in two ways from conductance data: from the slope of a plot of the logarithm of association constant K_A against reciprocal dielectric constant, and from $J(a)$, the coefficient of the linear term in the mobility. Data are available for tetrabutylammonium bromide in three quite different systems for testing this prediction. In dioxane-water mixtures, $\Sigma = 5.55$, $a(K_A) = 4.94$ and $a(J) = 5.0$–5.4. In methanol-carbontetrachloride, $\Sigma = 5.75$, $a(K_A) = 5.45$, $a(J) = 5.7$–6.0. In nitrobenzene-carbon tetrachloride, $\Sigma = 4.90$, $a(K_A) = 6.1$–6.6, $a(J) = 5.45$. The close agreement among these values suggests that the "ion size," long a catch-all for unknown terms in the conductance function, can now be considered a parameter characteristic of a given ionophore. If equation (9) turns out be be general, one of its most useful consequences will be the evaluation of limiting conductances in solvent mixtures of low dielectric constant where none of our present $\Lambda - F(c)$ extrapolation methods are applicable. Furthermore, the extrapolation methods[6] now used can be considerably simplified if J can be evaluated from Λ_0; the conductance equation will be reduced from a 3-parameter equation to a 2-parameter equation.

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† Contribution No. 1540 from the Sterling Chemistry Laboratory.

‡ A plot of RD against D, rather than R against $1/D$, is used to test equation (9) for two reasons: (1) it gives more weight to the points at higher dielectric constant where the precision in Λ_0 is usually higher and (2) it spreads the small systematic deviations in a more uniform manner.

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*MECHANISM OF CONTRACTION IN THE MUSCLE FIBER—ATP SYSTEM**

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NATIONAL BUREAU OF STANDARDS AND NATIONAL INSTITUTES OF HEALTH

Communicated by Paul J. Flory, April 17, 1959

Introduction—Crystalline macromolecular systems which are axially oriented possess the inherent property of contracting when subjected to conditions conducive to melting. The shrinkage that accompanies the melting process is due to the diminished axial extension of polymer chains in the amorphous or molten state as compared with that of the oriented crystalline state. Anisotropic dimensional changes, as well as changes in thermoelastic properties that occur when fibrous natural rubber,[1,2] fibrous polyethylene,[3,4] and collagen[5,6] undergo melting have been recently discussed from this point of view. The shrinkage that is widely observed among fibrous proteins may be induced by a variety of chemical reagents. While the specific action of these various reagents may differ considerably, melting of the native crystalline polymer arrangement appears to be common to all processes of this nature.[4] If melting has been carried to completion, spontaneous re-extension of the fiber may be subsequently observed under conditions favorable to recrystallization only if the system contains a sufficient number of permanent intermolecular crosslinkages which survive the melting process.

Pryor[7] and Flory[8,9] have suggested that muscular contraction is also the result of the melting or partial melting of axially oriented crystalline regions composed of polypeptide chains. The polymer chains comprising the muscle myofibrils are known to exist in the highly oriented crystalline state[10] so that the first requirement for contraction to occur by this mechanism is satisfied. The postulated change in crystallinity with contraction has not been satisfactorily demonstrated and the experiments reported herein were undertaken for this purpose. Specifically, the change in length of glycerol-extracted rabbit psoas muscle upon immersion in ATP solutions of various concentrations at room temperature were investigated. In addition, the wide angle X-ray diffraction patterns at various stages of contraction have been correlated with the dimensional changes.

Experimental.—Glycerol-treated muscle fibers were prepared from rabbit psoas muscle following the method of Szent-Gyorgyi,[11] and stored at $-20°C$. Strips about thirty to forty mm long and about 1 mm thick were washed thoroughly in water to remove the glycerol. The length in water was measured using a scale that could be read to 0.5 mm. The fiber was then immersed in a 0.1 *M* KCl solution of the desired ATP concentration, for fifteen to thirty minutes. The fibers were periodically transferred to fresh solution during this period. After contraction in the ATP solution, the specimens were returned to water and the new length was measured. The ratio, in water, of the shrunken length to the original length was designated as L/L_0.

Flat-plate wide angle X-ray diffraction diagrams of native and contracted muscle fibers were taken with Ni-filtered Cu radiation (40 Kv., 20 ma.). The film to specimen distance was 6 cm. The exposure times ranged from four to seven hours. For reasons to be discussed below it was necessary that the fiber be kept wet for the entire exposure time. The fiber was mounted with its axis vertical and a very slow stream

of water, or ATP solution, was introduced at its upper end and allowed to flow over and through the specimen. Immersion of the lower end of the fiber in a bath of the same fluid permitted permeation by capillary action. Thus, the portion of the fiber exposed to the X-ray beam was kept saturated with water, or aqueous solution, without interposing an enclosure.

Results and Discussion.—The relative changes in length are plotted in Fig. 1 against the ATP concentration of the solution. The general shape of the curve in this figure is very similar to that obtained by correspondingly plotting the similar experimental data previously reported by Bowen.[12] In his experiments, however, the magnitude of the contraction at each ATP concentration is somewhat larger than reported here, a fact attributable to the absence of Mg^{++} ions in the shrinkage solution used in the present work. The major portion of the observed contraction is restricted to a very narrow range of ATP concentration. This is the most significant feature of Figure 1. At concentrations of ATP greater than 0.003 *M* the shrinkage reaches a maximum value; at concentrations less than 0.001 *M* contraction is barely perceptible. Moreover, slight extension may occur when the specimens are returned to water from solutions of 0.0015 *M* or less in ATP.

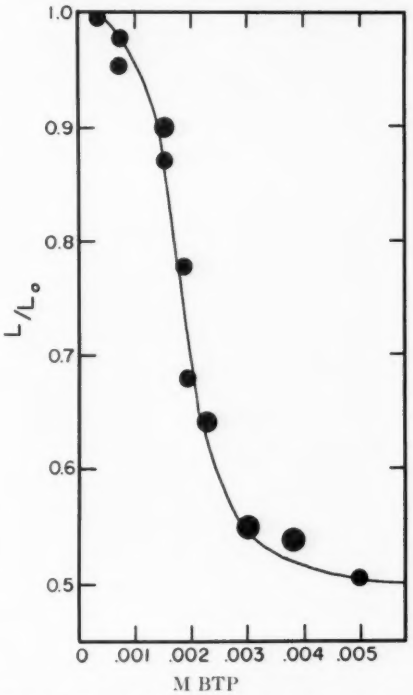
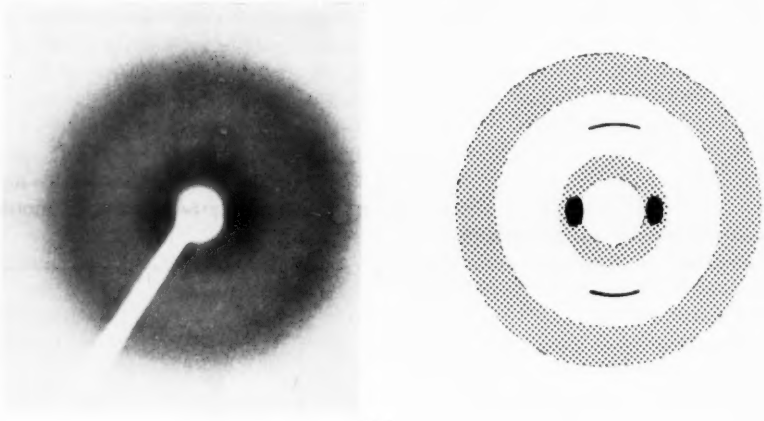


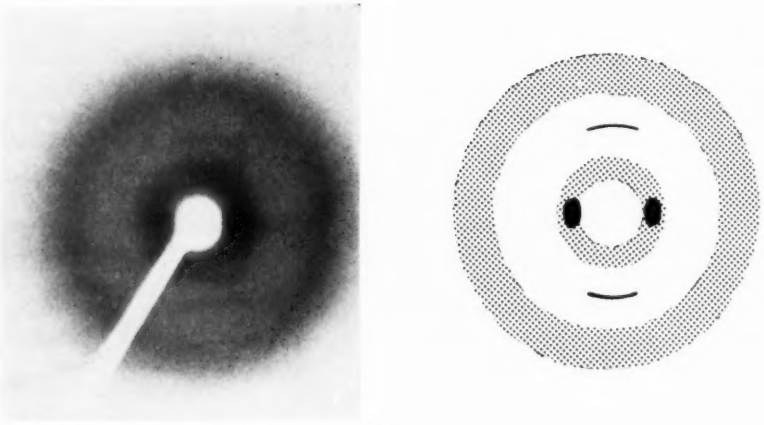
FIG. 1.—Plot of quantity L/L_0 against concentration of ATP.

The curve of Figure 1 is indicative of cooperative phase transition. Only a slight variation of the thermodynamic environment is required to induce the transformation. The length in this instance can be regarded as a thermodynamic variable. For the previous systems studied,[1,4,5] melting has been accomplished by increasing the temperature and the transformation occurred over a narrow temperature interval. In the muscle fiber-ATP system melting is induced isothermally by an appropriate chemical reaction. However, the underlying mechanism of the contraction is still the same for both types of process.

Although the characteristic curve of Figure 1 gives substantial evidence that contraction and melting are concomitant processes for the system under study, evidence for structural changes that might occur during the contraction were also sought. To accomplish this, wide angle X-ray diffraction patterns of the fibers, wetted with water, after various degrees of contraction were obtained and some typical results are illustrated in Figure 2. Although X-ray scatter by air and water are present in



(a)



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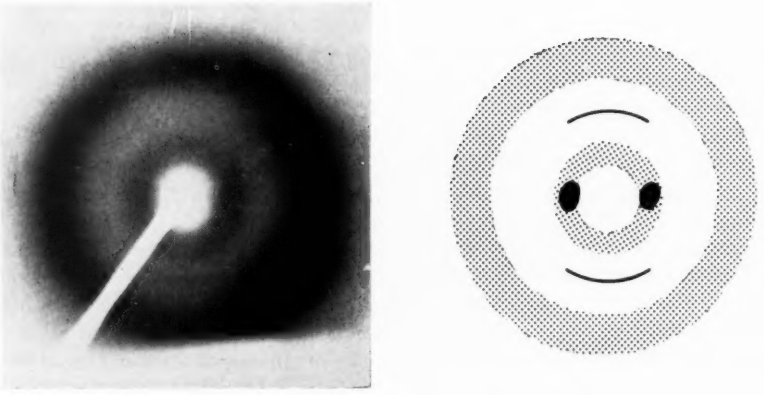
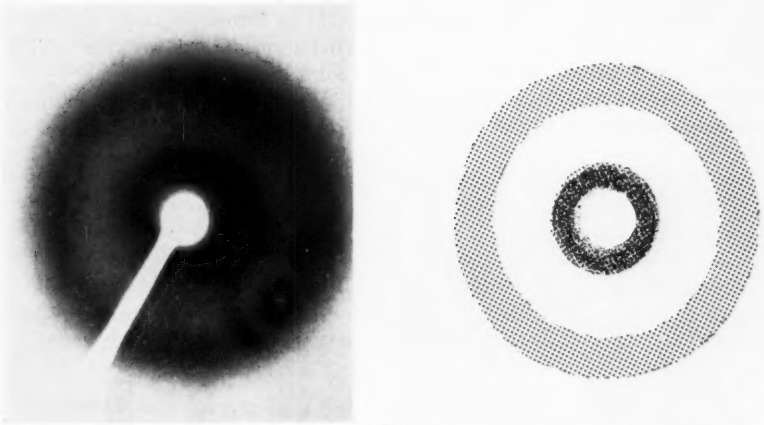


(c)



(d)

FIG. 2.—Wide angle X-ray diffraction patterns of glycerol extracted rabbit psoas muscle fibers. Fibers maintained moist with water. (*a*)Native fiber. (*b*) After immersion in 0.00150 *M* ATP solution. (*c*) After immersion in 0.00188 *M* ATP solution. (*d*) After immersion in 0.00375 *M* ATP solution.

these patterns the major structural features and changes that occur are easily discerned, and are shown in each case by an accompanying idealized diagram. The pattern for the native fiber, illustrated in Figure 2a, is typical of that given by an oriented α-keratin-type of structure as previously reported by Astbury.[10] The major features of this pattern are the 5.2 A meridional reflection and the intense equatorial spacing at 9.9A.

This pattern (Fig. 2a) is indistinguishable from that of Figure 2b in which case the fiber had been immersed in 0.00150 *M* ATP solution and then returned to water before the X-ray diffraction examination. Thus the structural changes accompanying the diminution in length developed by this treatment are not detectable by X-ray diffraction.

When, however, the fibers were shrunk in 0.00188 *M* ATP solution changes were observed in the resulting X-ray patterns, as is illustrated in Figure 2c. The meridional arc at 5.2 A has become more extended; the equatorial reflection displays a similar change. These results indicate the onset of destruction of the ordered arrangement of the chain elements.

The pattern of Figure 2d illustrates quite vividly the marked structural changes that occurred in the system at the termination of the process. The 5.2 A reflection has disappeared and in place of the intense 9.9 A equatorial spots originally observed a broad halo has appeared. Thus, the X-ray diffraction studies yield results which are in close accord with the major conclusion drawn from Figure 1, namely, that contraction is a consequence of melting. Only the melting of the polypeptide chains involved in the α-keratin-like structure has been observed; the characteristic wide angle diffraction pattern of the actin component[13,14] is not resolved.

The structural changes that accompany the contraction are still in evidence when the fibers are maintained moist with the same ATP solution utilized in shrinkage,

rather than with water. This fact is illustrated in Figure 3a where the fiber was contracted in a 0.00375 *M* ATP solution and maintained moist with the same solution. A pattern almost identical to that of Figure 2d is obtained.

The necessity for maintaining the fibers moist during the X-ray diffraction study is illustrated by the pattern in Figure 3b. In this instance the specimen was delib-

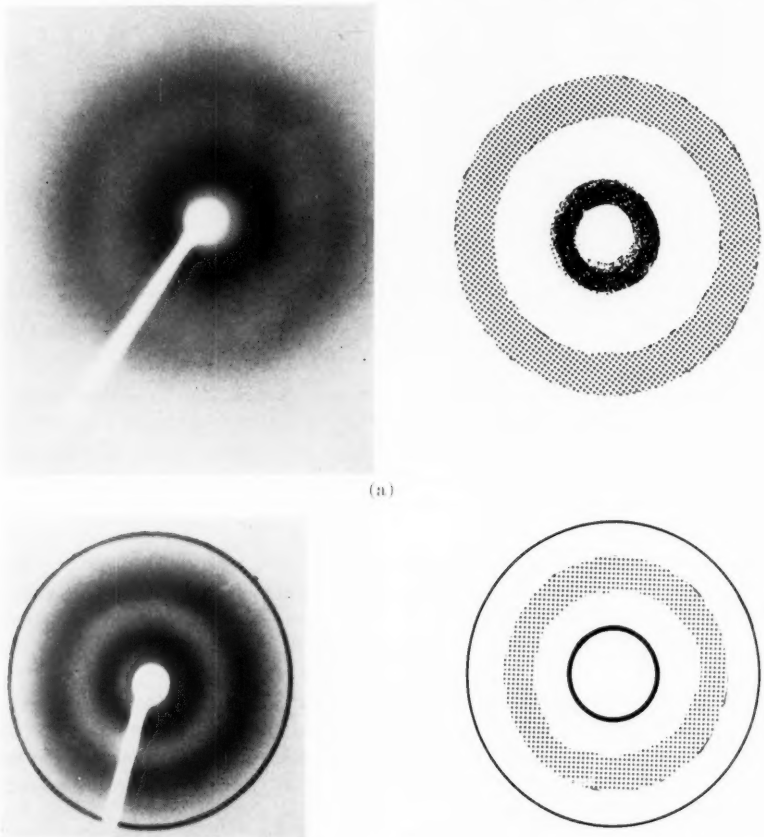


FIG. 3.—Wide angle X-ray diffraction patterns of glycerol extracted rabbit psoas muscle fibers. (*a*) After immersion in 0.00375 *M* ATP solution and maintained moist with this solution. (*b*) After immersion in 0.00375 *M* ATP solution and air dried. The outer sharp maximum is due to KCl.

erately allowed to air dry after shrinkage in 0.00375 *M* ATP solution. This figure shows the presence of a reasonably sharp halo in the vicinity of 9.9 A. This ring could be interpreted as resulting from a partial random crystallization due to drying. Whatever the interpretation, it is obviously necessary to keep the fiber moist when studying the contraction by X-ray diffraction.

The experimental results discussed above give strong support to the previous sug-

gestions[7-9] concerning the physiological mechanism of muscle contraction. Although a significant diminution in length may result from partial melting which is not easily detected by X-ray diffraction, the continuity of the contractile process of Figure 1 indicates that the same mechanism must be responsible for the shrinkage over the entire concentration range. Astbury[10] observed an increase in the angular dispersion of the 5.2 A meridional reflection upon stimulated contraction of living byssus retractor muscles. The contractures in his experiments were limited to 58 per cent; further stimulated contraction would lead to the "delta" state of Ramsey and Street[15] with its very different physical properties, which are typical of the amorphous state. Although the development of a high degree of shortening may go beyond actual physiological conditions, the highly contractive state is nevertheless of prime significance from an interpretative point of view, for, as has been stressed recently by Szent-Gyorgyi,[16] this state is developed in a continuous fashion from native muscle.

* This investigation was supported in part by Research Grant D-572, U. S. Public Health Service.

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*PARAMUTATION AT THE R LOCUS IN MAIZE PLANTS TRISOMIC FOR CHROMOSOME 10**

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Communicated April 6, 1959

Previous experiments have shown that the aleurone pigment-producing action of the R^r allele in maize, which gives darkly mottled kernels following testerosses of the standard R^rR^r strain on r^gr^g females (colorless aleurone, green seedling) is greatly reduced in all the $R^rr^gr^g$ kernels from r^gr^g ♀ × R^rR^{st} ♂ (heterozygous stippled and r^gr^g ♀ × R^rR^{mb} ♂ (heterozygous marbled) matings.[1-3] The altered forms of R^r are metastable, and revert toward standard R^r in homozygotes and heterozy-

gotes with colorless alleles. Complete reversion, however, has not been observed in any instance. No change in the R^{st} and R^{mb} progeny from the heterozygotes with R^r has been detected.

The process by which the heritable change in R^r action arises is called paramutation. In accordance with this terminology, R^r is paramutable, and the stippled and marbled alleles may be said to be paramutagenic.

The earlier evidence, as implied above, demonstrated that the alteration in R^r phenotype observable among the offspring of R^rR^{st} plants is based upon genetic change occurring in the parent heterozygote at, or close to, the R^r locus in the long arm of chromosome 10, in response to the action of an element contained in, or adjacent to, the R^{st} allele. The process thus involves some sort of interaction between factors at corresponding positions in homologous chromosomes. In view of this fact, it might reasonably be supposed that standard R^r changes to R′ (a convenient symbol for the paramutant form of R^r) in R^rR^{st} plants at zygotene in meiosis, when the chromosomes are conjugated side by side, so as to bring the R^r and R^{st} alleles into physical contact with each other. Somatic chromosome pairing has not been observed in maize. If, therefore, propinquity of the two homologous chromosome parts concerned is significant for paramutation at all, it is to the zygotene stage of meiosis that one must look for a regular effect of this factor.

The results of an experiment to test the validity of the hypothesis that paramutation occurs at zygotene, when the R^r and R^{st} alleles are synapsed, are summarized in the present paper.

Newton and Darlington[4] observed that chromosome pairing at zygotene in triploid and various aneuploid hyacinths and tulips was 2 by 2 at any given level. If such 2 by 2 conjugation holds regularly when more than two chromosomes of a given kind are present in the nucleus of a sporocyte, then suitably marked maize plants trisomic for chromosome 10 should provide material for critically testing whether paramutation is dependent upon zygotene pairing.

Trisomic maize plants appropriate for such a test would carry standard R^r in one chromosome 10, R^{st}, a paramutagenic allele, in the second member of the set, and a nonparamutagenic allele, such as r^g, in the third chromosome 10 ($R^r/R^{st}/r^g$). From such plants, only gametes carrying R^r ex-conjugants from R^r-R^{st} zygotene pairings would be expected to be paramutant, on the hypothesis in question. Any gamete carrying an R^r allele that had paired with r^g, or had remained unpaired at zygotene, should be of the standard type. Thus two classes of R^r gametes would be expected from $R^r/R^{st}/r^g$ trisomic individuals, paramutant and standard. The former should coincide with the paramutant R^r gametes invariably formed by ordinary, disomic, R^rR^{st} plants, and the latter with the R^r gametes arising regularly from standard R^rr^g individuals.

Pollen grains containing nuclei with $n + 1$ chromosomes rarely function in maize, and so may be ignored in predicting the outcome of disomic female × trisomic male matings. Assuming trivalent formation and random disjunction of the three chromosomes 10 at meiosis it would be expected, on the hypothesis under test, that $R^r/R^{st}/r^g$ plants would produce R' and R^r functional microspores in a 1:2 ratio. In so far, however, as the three homologous chromosomes in meiosis form pairs, the members of which then separate regularly, and the univalent chromo-

some is distributed at random to the daughter nuclei, the proportion of R^r and R' microspores will tend to approach 1:1. It may be predicted, therefore, that not more than one-half, and possibly as few as one-third, of the functional R^r male gametes from $R^r/R^{st}/r^g$ plants would be of the paramutant class, if zygotene pairing between R^r and R^{st} is a condition of paramutation of R^r. All the R^r gametes formed by R^rR^{st} (disomic) individuals on the other hand, are known to be of the R' type.

Materials and Methods.—The R^rr^g, R^rR^{st}, and $R^r/R^{st}/r^g$ sib plants that were used eventually as pollen parents in testcrosses on r^gr^g females were produced in a series of matings based upon a stock trisomic for chromosome 10 that was kindly supplied by Dr. Margaret Emmerling. The R alleles present in the original trisomic culture were R^{nj} (Navajo, conditioning aleurone pigmentation in the crown portion of the kernel), R^{st}, and r^g. The R^{st} chromosome also carried a large heterochromatic knob (K) distal to, and closely linked with, the R locus. Rhoades[5] has shown that plants heterozygous for K and contrasting R alleles show preferential assortment for chromosome 10 in macrosporogenesis, leading to a large excess of eggs carrying the knobbed chromosome, and to correspondingly distorted R phenotypic ratios. Since it was not known whether the knob would also affect meiotic conjugation and paramutation at the nearby R locus, the knobbed chromosome was replaced in the trisomic stock with a knob-free R^{st} chromosome from the appropriate W22 inbred line in our cultures. The substitution was effected by visually selecting for propagation the kernels showing both the Navajo and stippled aleurone patterns (superposed on each other) arising after pollination of foundation $R^{nj}R^{st}(K)r^g$ plants by W22 $R^{st}R^{st}$. Among the plants reared from these exceptional seeds, and then pollinated by W22 r^gr^g, those forming Navajo, stippled, and colorless kernels were recognized as the desired R^{nj}/R^{st} (knob-free)$/r^g$ individuals.

Plants grown from $R^{nj}/R^{st}/r^g$ seeds on the above ears were then used as pistillate parents in crosses with the standard W22 R^rR^r strain. Families were reared the next year from random samples of seed on four ears resulting from such $R^{nj}/R^{st}/r^g$ ♀ × R^rR^r ♂ matings.

None of the several kinds of progeny arising from this cross can be recognized with certainty at the flowering stage, although the trisomic plants tend both to be shorter than the disomic individuals, and to have lighter red anthers. The offspring, therefore, were numbered, and each was pollinated with W22 r^gr^g, with a view of post-harvest identification of the genotype. Likewise, pollen from each plant was applied to the silks of an unrelated r^gr^g inbred line, known as W23, that had been found previously to be especially suitable for differentiating between the R^r and R' aleurone phenotypes if the R allele is present in single dose ($R^rr^gr^g$ or $R'r^gr^g$). The ear progenies subsequently scored in detail for aleurone pigmentation were those resulting from pollination of W23 r^gr^g plants by R^rr^g, R^rR^{st}, and $R^r/R^{st}/r^g$ males.

All four families used in the latter testcrosses gave similar results. Since the total number of ears harvested was considerably in excess of requirements, however, further attention was limited to a single family, 45–417, which was found to contain the largest number (9) of $R^rR^{st}r^g$ plants, the least frequently occurring class of segregates involved in the comparison. From the relatively large numbers of testcross ears involving 45-417 R^rr^g and R^rR^{st} male parents, seven of the first

kind, and nine of the second were chosen at random for scoring, as controls.

The aleurone phenotypes resulting from R^r action in single dose in the triploid endosperm ($R^r r^g r^g$) are mottled, and variable. Differences in residual genotype contribute to the diversity in distribution and intensity of pigmentation over the kernel surface. This source of variation was partially controlled in the present experiment by virtue of the fact that three successive matings to highly inbred W22 lines were involved in preparing the trisomic stock. Furthermore a second, very uniform, inbred line, W23, was used as the pistillate parent in the ultimate testcrosses.

The kernels resulting from the application of pollen from 7 $R^r r^g$, 9 $R^r R^{st}$, and 9 $R^r/R^{st}/r^g$ plants in family 45-417 to W23 $r^g r^g$ females were scored for aleurone pigmentation under a binocular microscope fitted with a 20 × 20 reticule that gave a total coverage of about 12 mm² at ×30 magnification. Only the two top rows, comprising 40 squares, on the reticule scale were read. This reduced the kernel area sampled to about 1.2 mm². The particular portion of the kernel scanned in each case was a more or less uniformly elevated portion on the slightly dented crown characteristic of W23 seeds, near the point of silk attachment. Eighty R^r kernels were scored from each testcross ear. The scores are reported on a scale of 0–40, corresponding directly to the reticule reading in terms of the number of squares containing one or more pigmented aleurone cells.

The $R^r r^g r^g$, $R^{st} r^g r^g$, and $r^g r^g r^g$ testcross kernels formed an overlapping series of aleurone phenotypes. Only the young seedlings from kernels carrying the R^r allele, either in standard or paramutant form, however, develop anthocyanin; $R^{st} r^g$ and $r^g r^g$ seedlings are green. After scoring for aleurone pigmentation, therefore, each kernel was placed in order in a germinating pan, and the seedling color was noted following germination in the light. The R^r scores for each testcross ear were then based upon the first 80 kernels in the germinating pan that gave red seedlings.

A different kernel scoring procedure was used in the incidental comparison made to determine whether the previously untested Navajo allele was paramutagenic in $R^r R^{nj}$ heterozygotes. In this case, the readily distinguishable $R^r r^g r^g$ seeds resulting from W23 $r^g r^g$ ♀ × $R^r r^g$ ♂ and W23 $r^g r^g$ ♀ × $R^r R^{nj}$ ♂ matings were scored, with ×2.5 magnification, against a set of seven standard kernels varying progressively from no pigmentation, through four grades of mottling, to self-color. This procedure is rapid, and gives definitive results in such instances as the present.

TABLE I

DISTRIBUTION OF GENOTYPES IN FAMILY 45-417, RESULTING FROM A $R^{nj}R^{st}/r^g$ ♀ × $R^r R^r$ ♂ MATING

Genotype	Number of Plants
Disomic	
$R^r r^g$	41
$R^r R^{st}$	37
$R^r R^{nj}$	33
Trisomic	
$R^r/R^{st}/r^g$	9
$R^r/R^{st}/R^{nj}$	10
$R^r/R^{nj}/r^g$	7
Total	137
Per cent trisomic offspring	19.0

Experimental Results.—Distribution of offspring from a $R^{nj}/R^{st}/r^g$ ♀ × R^rR^r ♂ mating: Shown in Table 1 are the frequencies of the six genotypes observed in family 45-417, resulting from pollination in the preceding generation of a $R^{nj}/R^{st}/r^g$ trisomic plant with the standard W22 R^rR^r stock. Classification of the offspring was based upon the kernel phenotypes resulting from (*a*) pollination of the 45-417 plants with W22 r^gr^g males, and (*b*) matings on W23 r^gr^g females of the respective 45-417 individuals used as males. Both sets of testcross ears proved usable for determining genotype, and the two independent sets of observations were concordant throughout.

The occurrence of only 19.0 per cent, rather than 50 per cent, of trisomic plants among the offspring of the trisomic pistillate parent plant shows that there is strong selection at one or more stages in the reproductive cycle against cells in the female line carrying an extra chromosome 10, as has been observed by others for trisomics in maize, in general. The deficiency of the $2n + 1$ classes of offspring was of no consequence for the present experiment, however, except as the number of $R^r/R^{st}/r^g$ individuals available for the paramutation test was thereby reduced.

More significant is the fact that the three *R* alleles present in the trisomic pistillate parent of family 45-417 were transmitted to the offspring with nearly equal frequency. The distribution, based on the data in Table 1, is 57 r^g, 56 R^{st}, and 59 R^{nj}. Evidently there was no selection in the reproductive process favoring any one of the homologous chromosomes thus marked. It will be noted also from Table 1 that the three classes of trisomic offspring in family 45-417 occurred with approximately equal frequency.

Testcrosses on W23 r^gr^g females of R^rr^g segregates: Eighty R^r kernels from each of the ear families representing the testcrosses of seven randomly chosen R^rr^g plants in family 45-417 on the W23 r^gr^g line were scored for aleurone pigmentation. The results are summarized in Table 2. The average score proved to be 39.23, indicating

TABLE 2

MEAN ALEURONE PIGMENTATION SCORES BASED UPON 80 R^r KERNELS EACH FROM THE TESTCROSS EARS OBTAINED BY POLLINATING W23 r^gr^g FEMALES BY SEVEN RANDOMLY CHOSEN R^rr^g SEGREGATES IN FAMILY 45-417

R^rr^g Parent	Mean Score
45-417-8	39.69
-12	39.61
-42	38.79
-65	38.98
-94	39.61
-105	38.63
-128	39.30
Average	39.23 ± 0.163

heavy aleurone pigmentation following the testcrosses of this class of segregates on r^gr^g females. Likewise, as the individual entries in the table show, there was little variation in the mean color score from one R^rr^g male plant to another.

No ears from matings on W23 r^gr^g females of standard W22 R^rr^g plants (i.e., from crosses between the stock inbred lines W22 R^rR^r and W22 r^gr^g) were included among those scored in the present experiment. Casual examination of $R^rr^gr^g$ kernels produced in this way for other purposes shows, however, that they are only slightly darker, on the average, than those from the W23 r^gr^g ♀ × R^gr^g (45-417) ♂ testcrosses. The deviation is no greater than might well have arisen from

the difference in residual inheritance between family 45-417 and the standard R^rR^r line. It may be concluded, therefore, that the R^rr^g segregates in family 45-417 yield an essentially standard $R^rr^gr^g$ phenotype.

Testcrosses on W23 r^gr^g females of $R^r/R^{st}/r^g$ (trisomic) and R^rR^{st} (disomic) segregates: The data are assembled in Table 3 from the two classes of testcrosses

TABLE 3

FREQUENCY DISTRIBUTIONS OF ALEURONE PIGMENTATION SCORES FOR THE R^r KERNELS ONLY OBTAINED ON POLLINATING W23 r^gr^g FEMALES BY (*a*) R^rR^{st} AND (*b*) $R^r/R^{st}/r^g$ SEGREGATES IN FAMILY 45-417

Male Parent	Class Values for Aleurone Pigmentation of R^r Progeny									Mean Score*
	0	3	8	13	18	23	28	33	38	
	R^r Kernels Only From r^gr^g ♀ × R^rR^{st} (Disomic) ♂									
45-417-6	49	26	5	..	..	..	..	..	..	1.18
-25	13	24	20	7	6	4	2	..	4	9.01
-41	10	23	17	11	7	4	1	..	7	10.61
-44	17	31	12	9	7	4	..	..	..	6.40
-57	31	31	7	6	1	2	..	..	2	4.26
-74	47	22	7	3	1	..	..	..	..	2.03
-100	7	34	15	7	6	9	..	2	..	8.49
-110	58	21	..	..	1	..	..	..	..	0.74
-137	44	20	9	4	1	1	1	..	..	2.84
	Average R^r score for disomic testcrosses									5.07 ± 1.23
	R^r Kernels Only From r^gr^g ♀ × $R^r/R^{st}/r^g$ (Trisomic) ♂									
45-47-35	44	22	5	6	2	1	..	..	..	2.98
-50	37	31	6	2	1	1	..	..	2	3.51
-67	23	27	13	8	4	2	2	1	..	5.84
-84†	30	25	9	5	5	1	..	..	2	5.01
-85	21	22	8	9	4	4	2	2	8	10.33
-90	17	21	10	9	2	2	..	1	18	13.05
-107	23	28	10	7	4	3	3	2	..	6.63
-115	33	34	6	1	2	3	..	..	1	3.28
-126	20	33	10	4	3	5	3	2	..	6.71
	Average R^r score for trisomic testcrosses									6.37 ± 1.13‡

† 77 kernels only.
‡ Difference between average scores for R^r kernels (trisomic-disomic) = 1.30 ± 1.67.
* The values in this column are averages based upon the individual kernel scores, rather than upon the data after grouping into the classes shown.

on r^gr^g females that provide a critical basis for determining whether the addition of a third chromosome 10, bearing the nonparamutagenic r^g allele, to R^rR^{st} heterozygotes, used as pollen parents, alters the phenotype of the $R^rr^gr^g$ offspring. The lower half of the table embodies the results from the nine trisomic segregates found in family 45-147 that carried the R^r, R^{st}, and r^g alleles. Shown in the upper half of the table are the data from an equal number of disomic R^rR^{st} sibs randomly chosen from the 37 plants of this class that were successfully tested on the W23 line. The figures entered in the table relate to the $R^rr^gr^g$ testcross kernels only, identified as such by the red seedlings they produced.

It will be observed at once that the mean aleurone pigmentation scores for the $R^rr^gr^g$ ($R^rr^gr^g$) testcross kernels in both the disomic and trisomic groups in Table 3 are far below those for the corresponding class of kernels resulting from the r^gr^g ♀ × R^gr^g ♂ matings referred to in Table 2. The observed values are 5.07 ± 1.23 for the disomic R^rR^{st} controls, and 6.37 ± 1.13 for the $R^r/R^{st}/r^g$ trisomic plants, as compared with 39.23 ± 0.16 from the R^rr^g sibs, similarly tested. Evidently, therefore, R^{st} is strongly paramutagenic in R^r heterozygotes whether or not the R^r and R^{st} chromosomes are accompanied in the sporocyte nucleus by a third chromosome 10 carrying the nonparamutagenic allele, r^g.

Also apparent from Table 3 is the fact that the average scores for aleurone pigmentation of the $R^r r^g r^g$ testcross kernels in the $R^r R^{st}$ and $R^r/R^{st}/r^g$ groups are essentially alike. The difference between the two means, 1.30 ± 1.67, is no greater than would frequently be expected from random sampling.

Furthermore, the two groups of frequency distributions for aleurone pigmentation in Table 3 are of the same general form. Throughout all families, most of the $R^r r^g r^g$ testcross kernels were either colorless or only weakly colored in the predetermined area that was scored, and the number of kernels assignable to the progressively darker classes fell off rapidly.

The seemingly excessive number of entries in the top class for kernel pigmentation in a few instances (notably plant 45-417-90) could have resulted from contamination by standard R^r pollen from an outside source, even though operating conditions were favorable when the pollinations were made, and the usual precautions were taken to minimize this source of error. These exceptional kernels occur in both the disomic and trisomic groups, and since they are known, on the basis of numerous other tests, not to be characteristic of testcrosses involving $R^r R^{st}$ plants, they may reasonably be considered extraneous in the case of the trisomic individuals also.

It may be concluded from the data in Table 3 that the R^r male gametes formed by otherwise similar disomic $R^r R^{st}$ and trisomic $R^r/R^{st}/r^g$ plants are alike throughout in having a much reduced aleurone pigment-producing potential.

The nonparamutagenic action of the Navajo allele in $R^r R^{nj}$ heterozygotes: The presence in family 45-417 of 33 $R^r R^{nj}$ plants provided an opportunity to test whether the Navajo allele was paramutagenic in this genotype. The $R^r r^g r^g$ kernels from testcrosses on W23 $r^g r^g$ females of seven such heterozygotes were scored for aleurone pigmentation against a set of standard kernels varying in degree of mottling. The corresponding class of seeds from a like number of $r^g r^g$ ♀ × $R^r r^g$ ♂ testcrosses, similarly scored, served as the controls in this experiment. The data, which are summarized in Table 4, give no indication that the Navajo allele is paramutagenic. (The reader's attention is directed to the footnote to Table 4 pointing out that, because of the differences in scoring method and scale used, the numerical values in this case are not comparable with those in Tables 2 and 3.)

Discussion.—The present experiment, designed to learn whether chromosome conjugation in meiosis is a condition of paramutation at the *R* locus in maize, was prompted by three earlier observations: (1) invariability of the change from R^r to

TABLE 4*

MEAN SCORES FOR ALEURONE PIGMENTATION OF 80 R^r KERNELS ON SEVEN EARS EACH, FOLLOWING POLLINATION OF W23 $r^g r^g$ FEMALES BY $R^r r^g$ AND $R^r R^{nj}$ SIBS IN FAMILY 45-417

$r^g r^g$ ♀ × $R^r r^g$ ♂		$r^g r^g$ ♀ × $R^r R^{nj}$ ♂	
Male Parent	Mean Score	Male Parent	Mean Score
45-417-8	3.05	45-417-1	2.44
-12	3.05	-17	2.49
-42	3.01	-51	2.26
-65	3.20	-86	3.48
-94	2.88	-122	3.25
-105	2.53	-134	2.88
-128	2.74	-141	3.43
Average	2.92 ± 0.084†	Average	2.89 ± 0.191†

* The aleurone pigmentation scores reported in this table are relative to those arbitrarily assigned to a set of standard kernels varying in grade of mottling, and thus are not comparable with the values given in Tables 2 and 3. See text.

† Difference between means = 0.03 ± 0.209.

R' among the testcross offspring of R^rR^{st} heterozygotes, (2) localization of the genetic alteration at, or close to, the R^r locus in one chromosome of a pair, in response to the action of a second element closely associated with the stippled allele at the same level in the homologous chromosome, and (3) the relative uniformity of the R' paramutant phenotype. All these facts are compatible with the view that paramutation occurs under a specific set of conditions that are especially advantageous for an interaction between allelic factors. This reasoning led to the postulate that R^r is changed to R', in R^rR^{st} heterozygotes, when the chromosomes bearing R^r and R^{st} are conjugated in the sporocyte nucleus, so as to bring the two alleles into apposition with each other.

The experimental data obtained, however, negate the hypothesis that paramutation occurs when the chromosomes are conjugated at zygotene. The test applied was critical, assuming validity of the basic premise of Newton and Darlington that when more than two homologous chromosomes are present in a sporocyte nucleus only pairs associate at zygotene at any particular point. The test made would not be meaningful, however, if the three different parings at each level that are possible in a trisomic set of chromosomes occurred sequentially. The writer is unaware of any evidence that sequential pairing occurs in meiosis or, on the other hand, that positively excludes this possibility.

The alternative view, namely, that in R^rR^{st} plants R^r is changed to R' in somatic cells, is without direct experimental support at present. The relevant data, however, are few. Brink[1] observed that the kernels formed after $R^{st}R^{st}$ ♀ × R^rR^r ♂ matings are as darkly pigmented as would be expected from the action of two doses of the stippled allele, plus a single dose of the standard R^r allele, even though the $R'R^{st}R^{st}$ seeds resulting from $R^{st}R^{st}$ ♀ × R^rR^{st} ♂ crosses are obviously paramutant in R^r phenotype. This relation is even more clearly apparent after $R^{mb}R^{mb}$ ♀ × R^rR^r ♂ matings. In certain homozygous marbled sublines of the W22 inbred strain the marbled allele which, like stippled, is strongly paramutagenic in heterozygotes with R^r,[3] conditions the formation of only a few small spots of colored aleurone tissue. When such marbled plants are pollinated with standard R^r, the ensuing kernels are as darkly pigmented as those resulting from rr ♀ × R^rR^r ♂ matings. Evidently the pigment-producing action of standard R^r is not immediately impaired when R^r enters a nucleus at fertilization in which a paramutagenic allele is already present. This is true, at least, in the case of the endosperm mother nucleus, one of the two products of the double fertilization involved in reproduction of the maize plant.

The color determining action of the standard R^r allele in vegetative tissues of the W22 strain, which is dilute sun red, is restricted, for the most part, to certain seedling structures and to the glumes and anthers in the full grown plant. Very little anthocyanin is formed in the leaves and stem. Previous experiments have shown that paramutant R', derived from an R^rR^{st} parent, lowers pigmentation at both the seedling and adult stages in $R'r^g$ offspring.[6] It remains to be determined, however, whether a reduction in anthocyanin-producing action of the R^r allele can be detected in the R^rR^{st} parent heterozygote itself.

Meanwhile it should be noted that Coe[7] has recently reported a phenomenon involving the B locus in maize, resembling paramutation at the R locus in certain respects, that is clearly premeiotic. Another example of regular genetic change in

vegetative tissue, and referred to as "somatic conversion," has lately been described by Hagemann[8] in Lycopersicon. The cruciata allele (*cr*) in Oenothera, long studied by Renner,[9] and by Oehlkers,[10] and characterized by Renner as "conversion-active" in *Cr cr* heterozygotes, likewise appears to promote heritable changes in *Cr* in somatic cells.

Summary.—A comparison was made of the aleurone phenotypes of the $R^rr^gr^g$ kernels resulting from the following three matings in maize: (1) r^gr^g ♀ × R^rr^g ♂ (2) r^gr^g ♀ × R^rR^{st} ♂ (disomic), and (3) r^gr^g ♀ × $R^r/R^{st}/r^g$ ♂ (trisomic). The male parents were moderately inbred sibs, and the r^gr^g female parent was a highly uniform inbred strain. The object of the experiment was to test the hypothesis that paramutation of R^r to a weakly pigmenting form, conveniently designated R', which was known from previous observations to occur in 100 per cent of the cases in R^rR^{st} plants, takes place at the zygotene stage of meiosis, when the R^r and R^{st} alleles are synapsed. The r^g allele present in the R^rr^g and $R^r/R^{st}/r^g$ individuals had earlier been shown to be nonparamutagenic. Assuming 2 by 2 pairing at zygotene at any given chromosome level, only the R^r male gametes that carry R^r ex-conjugants from R^r - R^{st} zygotene pairings should be paramutant, and the rest should be normal, if the hypothesis in question is valid. Not more than 50 per cent, and perhaps as few as 33 per cent, of the R^r gametes formed by the $R^r/R^{st}/r^g$ trisomic plants should be paramutant on this basis. Aside from a few seeds on both groups of testcross ears that could have resulted from pollen contamination, it was found that the $R^rr^gr^g$ ($R'r^gr^g$) kernels resulting from the application to r^gr^g individuals of pollen from the trisomic $R^r/R^{st}/r^g$ plants, as well as from the disomic R^rR^{st} controls, were of the paramutant phenotype throughout. The r^gr^g ♀ × R^rr^g ♂ mating yielded the expected 50 per cent of standard $R^rr^gr^g$ seeds. The results of the experiment, therefore, negate the hypothesis that R^r is changed to the paramutant form, R', in R^rR^{st} plants, when the R^r and R^{st} alleles are conjugated at zygotene.

The technical assistance in this study of Dr. Bernard Mikula, Dr. Fredrick Valentine, and Mr. Bruce Ashman is gratefully acknowledged.

* Paper No. 721 from the Department of Genetics, College of Agriculture, University of Wisconsin. This study was aided by grants from the research committee of the Graduate School of funds supplied by the Wisconsin Alumni Research Foundation and by a grant from the National Science Foundation.

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*A REGULAR AND CONTINUING CONVERSION-TYPE PHENOMENON AT THE B LOCUS IN MAIZE**

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Communicated by Marcus M. Rhoades, April 8, 1959

It is extremely difficult to visualize a feasible process by which one molecule might transmute another into a copy of itself. In its original usage[1] the term *conversion* was concerned with changes in genes as abstract units; the assumption that *molecular* conversion was implied has come about with the development of molecular concepts for genic structure. As Goldschmidt points out,[2] "conversion" has been used to describe phenomena with effects that "do not fit into the classical conception of segregation and crossing over" in recent examples. No particular mechanism is implied in such usage—the term is thus applied in the same way as "position effect," "mutable locus," "crossing over," and "transformation."

This paper presents data on an effect in maize that is evidently of the conversion type, when considered in terms of phenotypic expression.

Materials and Observations.—At the *B* locus on chromosome 2, three known alleles,[3] *B*, B^w, and *b*, confer, respectively, intense, weak, and no anthocyanin color in the husks, sheaths, culm, and other less conspicuous plant parts. Dominance is in the same order but is not quite complete. This locus has not yet been given an extensive analysis for variability, compoundness, or mutation, but apparently there are other alleles, perhaps more complex in their relationships.

In 1953, two weak-colored plants were observed among approximately 140 in the uniform progeny of a single intense-sun-red (*B B pl pl*) individual. The two exceptions occurred separately, one on each of two selfed ears of one parent plant. Testing was obviously in order, for they were unusual from the first: if mutation of *B* to B^w had occurred, these exceptions would have had to be homozygous for B^w, but their frequency in the progeny did not suggest a segregation ratio; if a newly-arisen dominant weak (at any locus) had arisen, it would be a previously unobserved type.

The two exceptional plants were self-pollinated and crossed onto intense individuals of the same line. Figure 1 shows the combined results of these and subsequent pollinations. The self-pollinated progenies did not segregate for color but remained uniformly weak in color through three generations of selfing. Outcrosses to intense were all weak and continued to give only weak progeny throughout three generations of outcrossing as male and female to four widely different strains having intense color. Self-pollination of these outcrosses yielded no intense individuals.

Intense plants, *B*, have strong color in the husks, sheaths, culm, glume face, and glume bar; weak plants, designated as *B′*, have only a flush of color in these parts, occasionally none, except for the dark-colored glume bar at the base of the tassel glumes. *B* and *B′* are most conspicuously differentiated by husk color. Plants of *b b* constitution have no color in any of these parts, and are most clearly distinguished from *B′* by the absence of glume bar color.

As indicated in Figure 1, crosses of weak to green (*b b*) gave all weak plants in the

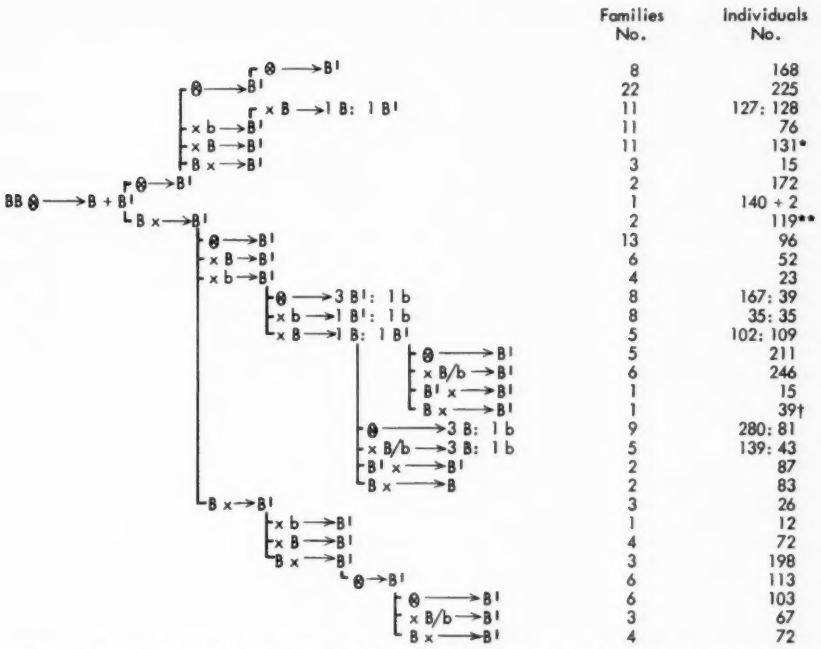

FIG. 1.—Progeny data for two exceptional maize plants with weak color and a continuing conversion-type effect. B = intense, B' = weak (converter), b = green plant color; "⊗" denotes self-pollination, "$B\times$" a cross on B B egg parent, "$\times$ B" by B B pollen parent, "$\times$ b" by b b pollen parent, etc. The numbers of individuals observed are given at the right for each progeny; the number of families indicates the number of progenies observed (individuals in the previous generation).

* Plus one having intense color but no ear and no pollen.

** One with a definite intense sector.

† Plus two intense (see text).

F_1; when these were selfed or backcrossed to b they segregated normally for dominant weak and recessive green. Crosses of the B' b F_1 to intense showed a ratio of 1 intense:1 weak; these crosses gave the only segregants having intense color in any progenies derived from B' plants. All weak plants of this background, when progeny-tested, showed only the B' type; intense plants consistently showed an absence of B' and a segregation for b. It is significant that chromosome 2 markers (lg_1, gl_2, v_4, Ch, inversion 2a) introduced with b have segregated normally, appearing as expected in the progenies.

Figure 1 may be summarized as follows: Two exceptional weak-colored individuals arose in a large progeny of intense plants; these exceptions failed to segregate for intense on selfing and continued to confer weak color in backcrosses to intense egg parents through three repetitions and to intense pollen parents in several crosses; crosses of weak plants to the phenotypic null, b, when selfed or backcrossed, segregated for b and associated markers; B' b individuals crossed to intense gave half weak plants, which were B' B' by progeny test, and half intense plants, which were B b. Other progenies shown in the figure give peripheral information that is entirely confirmatory.

The few exceptions to the pattern presented here are indicated by footnotes in Figure 1. A single intense plant appeared in a progeny from an outcross by *B* but was barren of ear and died without shedding pollen. It is assumed that this individual was intense because of the typical pigment increase in injured or barren plants. The one observed sector for intense, in an outcross progeny, is of considerable interest. This otherwise weak individual had an intense sector including approximately $^1/_8$ of the circumference of the sheaths, extending from a node near the ground through the internode sheath just above the top ear, but including neither ear nor tassel. Several smaller *possible* sectors have been observed in these progenies, especially from similar crosses, but the manner of expression of sun-red color makes it difficult to distinguish occasional true sectoring from a pattern of pigmentation due to growth and light effects. The rarity or absence of germinal exceptions, which are expected with regularity in unstable systems, suggests that the weak type is stable. Some preliminary observations on purple (*Pl;* alternative to sun-red) plants with the *B′* system indicate that small sectors occur at a low frequency. The only remaining exceptions to the general pattern are two intense plants in one family of 41 in which only *B′* was expected. The family concerned has reduced pollen fertility in several plants, including one of the exceptions. This would suggest a chromosome aberration of some kind. Further study of this family may clarify the origin of these exceptions.

To determine whether plant color in a *B′* background changes on repeated selfing or outcrossing to *B*, husk color grades were estimated in a 1958 planting of these progenies, on an arbitrary scale of 0–6 for increasing color. The grading was objective to the extent that the grader knew neither the backgrounds of the families nor the purpose of the experiment. The grades of *B* and *B′* families did not overlap: *B* plants ranged from 3–6 (mostly 4–5), and *B′* plants ranged from 0–2 (mostly 1). Table 1 shows the grade averages for these progenies. It would appear that no consistent changes occur. However, outcrossing to intense lines may maintain a slightly higher average level of color than does selfing. The outcross progenies show no indication of segregation for grade in the raw data, although distinguishing segregation among grades 0, 1, and 2 is difficult because of their close similarity.

TABLE 1

HUSK COLOR GRADES ON REPEATED SELFING AND BACKCROSSING OF WEAK-COLORED LINES

Source	No. Plants	Av. Grade
B comparator	38	4.6
B′ (X)	75	1.0
B′ (X) (X)	54	1.0
B′ (X) (X) (X)	160	0.9
B × B′	51	1.2
B × B/B′	7	1.9
B × B/(B/B′)	46	1.0

New occurrences of *B′* plants are observed occasionally in the original intense sun-red line, in frequencies varying from single exceptions in large families up to one-half or more of the progeny. In one case mutation of the whole family is suspected. Some derivatives of the original line appear to be stable; others seem to have a high potential for mutation to *B′* but do not have conspicuous weak sectors on the intense plants. An "ear map" of one *B′*-carrying progeny, in which the seeds were planted and observed according to their position on the ear, only vaguely

suggests the occurrence of sectors of B' on the ear, with varying sizes of "islands" of B' kernels. Preliminary tests on a few of these weak exceptions indicate properties parallel to those of the original B' individuals.

Discussion.—Segregates from $B'\ b \times B$ show a correlation of segregation for the weak color effect with segregation at the B locus on chromosome 2. In crosses of B' with B, however, normal segregation fails to occur, even when B' previously has gone through a generation of normal segregation with b. Known systems that can bring about failure of segregation, especially chromosomal transmission irregularities and cytoplasmic inheritance, should be explored.

If exclusive transmission of B' is involved, we must make the broad and improbable assumptions that B' is dominant for weak color, that exclusion of the B-carrying chromosome from the gametes is complete through both male and female, that no sterility occurs in connection with the exclusion, and that no exclusion of b gametes occurs. A simple test is in preparation, using a marked B chromosome to determine whether proper segregation occurs from heterozygotes with the B' chromosome. For the present it appears that a system completely associated with the B locus in which B' can exclude B but not b is very unlikely.

If a cytoplasmic factor is involved, it is rather unusual. It must be transmitted through both male and female with no reciprocal differences, be undiluted by repeated outcrossings as male, and have a remarkable relation to the nuclear genes in transmission—a plant carrying such a factor must produce B' gametes that bear the factor and b gametes that do not. These restrictions are not at all typical for cytoplasmically inherited effects in maize.[4] A complicated mechanism for chromosomal attachment and detachment of a "cytoplasmic" factor could be constructed but seems superfluous at present.

A comparison of these effects with those recently described[5] for certain alleles at the R locus in maize should be of interest. A striking similarity exists in the apparent interaction between alleles, and the basis for such interaction may very well be the same in the two cases. The two systems differ strikingly, however, in several ways. Changed R^r (that is, R' or $R^{r\text{-}st}$ or $R^{r\text{-}mb}$) is different from R^{st} or R^{mb}; B' is monotonously constant from change-to-change. R^{st} and R^{mb} are mutable; B' is relatively stable. When made homozygous, R' reverts very nearly to R^r; B' continues unchanged on selfing. Thus in the phenomenon presented here there appears to be a very specific induced change of B to B'—all the properties of B' are conferred upon B. In the R case the change that occurs is specific and may involve allele interactions, but the changed allele has few of the properties of its inducer. Indeed, the parallel relationship of changes in the two components[6] of this established compound locus indicates a mechanism distinct from the genes themselves, as does the reversibility of the change.[7]

The term *conversion*, originally applied by Winkler[1] to explain crossing over, has come to be discredited with later demonstrations of Mendelian explanations for apparent examples. Notwithstanding these clarifications, the term is useful, when held distinct from any implied mechanisms, in categorizing an unexplained system with apparent interaction between alleles. In view of the phenomena of transformation and transduction in microorganisms, in which introduced hereditary material transfers its properties into the hereditary material of recipient cells, it would seem likely that, within a diploid heterozygous nucleus (carrying two dif-

ferent species of DNA at homologous loci), examples of similar transfer could exist. Goldschmidt[8] recently applied such an explanation to an interesting case in Drosophila; he suggested a separate "transductor locus" to transfer a segment of chromosome from one homolog to the other. A distinct element which would presumably have similar properties was suggested by Brink[9] for the *R* case in maize. In the absence of indications for separability of such an element from *B′* by crossing over or transposition, or for multiple-locus action, no distinct element is implied in the system presented here. If such an element were invoked, a "transductor" would simply be a repository for the unusual effects at the *B* locus. Certainly a more likely mechanism would be one of enforced copying of *B′* as a property of *B′* itself, carried out at some time or times in the life cycle of *B′*/*B* plants. This is, of course, one method by which a phenotypic conversion could occur. A clear distinction should be drawn between conversion observed at the level of the whole life cycle of the organism and that observed at the level of replications of single chromosome strands or virus particles. However, conversion-type events observed in any system must be kept distinct from the mechanism[10, 11] used to explain them.

The striking features of the system presented here are the regularity (or infallibility) of the conversion effect, and the continuity of the effect from one life cycle to the next. Whatever their mechanism, the possibility should not be overlooked that conversion effects may occur, whether as infrequent exceptions for most loci or as the rule for certain loci. Maize should not be unique in this behavior; infrequent natural occurrence might be expected in any organism.

Summary.—An effect on plant color in maize, interpreted as a regular conversion of one allele at the *B* locus by another at some time or times in the life cycle, is reported. The effect is a continuing one in that the "converted" *B*, termed *B′*, is also regularly able to "convert" newly introduced *B*. The phenotypically null allele (*b*) is unaffected by *B′*.

The basic pattern is one of absolute failure of segregation when *B′* plants (weak color) are crossed repeatedly to *B* plants (intense color) as male or female, or when the *B′* individuals from such crosses are self-pollinated. Only when *B′* plants are crossed to *b* (green) and backcrossed to *B* do intense individuals appear. The intense individuals in these segregating progenies have received the chromosome 2 carrying *b*, and segregate *b*; the weak individuals have received the *B′* chromosome, and fail to segregate for *b* or *B*.

* Contribution from the Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, and the Missouri Agricultural Experiment Station. Jour. Series No. 1960.

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RECOVERY IN STATIONARY-PHASE PARAMECIA FROM RADIATION EFFECTS LEADING TO MUTATION

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Communicated by Alexander Hollaender, April 24, 1959

Recent studies[1-12] of mutation induction by ionizing radiation and ultraviolet light have indicated some sort of reversibility in the mutation process and have established the important facts that alterations in postirradiation metabolism can change the amount of mutation and that a time limit exists for this change. This time limit probably occurs before the first postirradiation division in most, if not all, cases.

The following terminology will be used to make it easier to discuss these phenomena. The damage produced by the absorption of the radiation and the rapid reactions that immediately follow it will be called *initial damage*. The term may include some mutation, i.e., inherited changes in the chromosomes, which are reversible only by further mutation if at all. It also includes damage that is potentially capable of leading to mutation but has not yet done so. This will be called *premutational damage* in preference to such a term as "potential mutation" because we wish to include all forms of radiation damage that might eventually cause or facilitate mutation, e.g., diffusible mutagens and metabolic disturbances as well as local lesions in the chromosomes. The period between irradiation and the time limit during which premutational damage may be influenced by postirradiation treatments will be called the *intermediate period*. We prefer this to Witkin's term[12] "sensitive period" since the latter has also been used in describing stage sensitivity. The premutational damage that is still present at the end of the intermediate period may be converted to mutation during what will be called, following Witkin,[12] the *terminal event* and any other damage that is still present after this event no longer has any influence on the amount of mutation.

Earlier work[5-7] has shown that recessive lethal and other deleterious mutations in *Paramecium aurelia* conform to this general pattern. Posttreatment with various metabolic inhibitors decreases the amount of mutation; there is a time limit (the terminal event) for this modification about midway through the interdivision interval. Furthermore, the amount of detectable mutation increases the nearer to the terminal event the radiation is given. These findings were interpreted to mean that premutational damage is lost during the intermediate period and that posttreatment agents act by increasing the time available for loss. There was no direct demonstration, however, of loss during the intermediate period; it was simply inferred from the increase of mutation with decrease of the time between irradiation and the terminal event on the reasonable but untested assumption that the amount of initial damage is not dependent on the time of irradiation during interphase. This paper shows that loss of premutational damage really does occur and gives some information about its rate. This was accomplished by irradiating paramecia in the stationary phase and varying the period between irradiation and transfer to fresh culture medium.

Materials and Methods.—*Paramecium aurelia* (variety 4, stock 51) individuals

undergoing the sexual process of autogamy were isolated and allowed to divide 10–12 times in limited quantities of culture medium (lettuce infusion inoculated with *Aerobacter aerogenes*). Five to seven days after isolation, samples were checked for autogamy and cultures with less than 1 per cent autogamy were selected; autogamy only rarely recurs so soon after a previous one.

The paramecia were irradiated in the exhausted culture medium in which they had been growing and kept en masse in this medium. At appropriate times after irradiation, animals were transferred to fresh culture medium, each to a separate container, and cultured thereafter by the daily isolation method.

Recessive lethal and other deleterious mutations were detected as in previous work. From each irradiated paramecium, 25 autogamous progeny were isolated 5–10 days after irradiation. The fraction of each group of 25 that survived with normal growth was recorded, and these fractions were transformed by a variance-equalizing transformation[7] to give a variable that decreases as mutation increases. This variable was subtracted from 2.4, the mean of a large series of unirradiated controls, in order to obtain a quantity, M, that increases as mutation increases. M increases nearly linearly with dose and so is probably directly proportional to the number of mutations, though the proportionality factor is unknown.

The X-ray source was a General Electric Maxitron 250 operated at 250 kvp, 30 ma, and with 3 mm of Al added filtration, giving a half-value layer of 0.43 mm of Cu. Before each run, monitoring was done with a Victoreen thimble chamber. The intensity varied from 1.5 to 1.8 kr per minute, and the total dose was 4.5 kr.

Results.—Figure 1 shows the results of three series of experiments in which the time between irradiation and transfer to fresh culture medium was varied. The paramecia were 5 days old in some experiments and 7 days old in the others. No

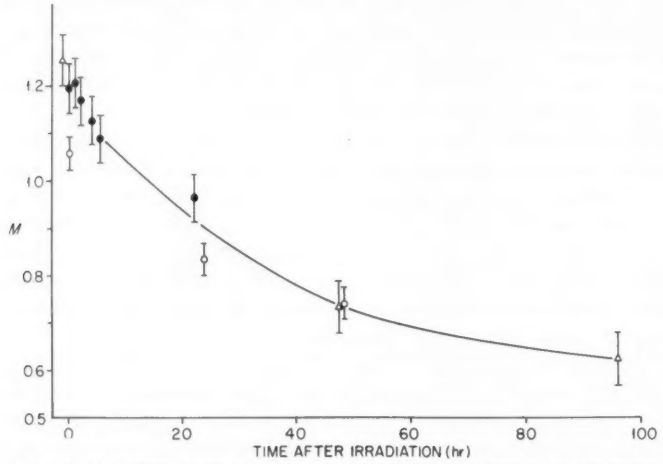

FIG. 1.—Decrease in mutation with duration of the postirradiation stationary phase $M \pm$ s.e. is plotted against time between irradiation and transfer to culture medium. The data are for several series of experiments done at different times and plotted separately.

●—Series 1, 3 experiments, 1 with 5-day-old and 2 with 7-day-old paramecia. ○—Series 2, 3 experiments, all with 7-day-old paramecia. △—Series 3, 1 experiment, 5-day-old paramecia.

differences that can be attributed to this variation in preirradiation starvation have been found in these or other experiments.

The data show a continuous decrease in mutation with duration of postirradiation starvation. The 24- and 48-hr points in the second series are significantly different at the 5 per cent level, showing that the decrease certainly continues for 24 hr, and there is no definite time at which the decrease stops. Instead, the curve appears to approach asymptotically to a lower limit of approximately 0.6.

Before the decrease in M in Figure 1 can be interpreted as a true loss of premutational damage, certain selection artifacts must be excluded. Since a dose of 4.5 kr is only 1–2 per cent of the dose needed to kill any vegetative paramecia, selective death during the period of holding en masse between irradiation and transfer to fresh medium is most improbable. Selective death could occur if autogamy took place during the postirradiation starvation since radiation-induced lethals could then become homozygous. This is an unlikely source of error, however, because (1) no increase in autogamy was observed, (2) death after autogamy usually occurs only after a postautogamous period of growth, and (3) only about 2 per cent of the paramecia that were transferred to culture medium failed to survive to produce autogamous progeny. This is too small a frequency to account for the decrease in detectable mutation; and, in any case, the percentage was nearly the same in the different time groups. Thus selective elimination of the more mutant animals by death before detection of mutation cannot be responsible for the results.

The existence of a real loss of premutational damage does not necessarily mean that the loss occurs during starvation. The additional starvation might change the conditions of the subsequent growth period in such a way that a greater loss would occur after transfer to fresh medium. If this were so, pre- and postirradiation starvation should be equally effective. The data in Table 1 show that an additional 2 days of preirradiation starvation had no effect. Therefore, the loss of

TABLE 1

LACK OF EFFECT OF ADDITIONAL PREIRRADIATION STARVATION ON MUTATION

Preirradiation starvation time, days	Postirradiation starvation time, days	$M \pm$ s.e.
5	0	1.35 ± 0.08
7	0	1.35 ± 0.08
5	1	1.03 ± 0.08
7	1	1.02 ± 0.08

premutational damage occasioned by postirradiation starvation must occur during the starvation period itself. This does not mean, however, that loss occurs only then. It is highly probable, from the earlier studies,[6] that loss of damage also occurs after transfer to culture medium, but the data show that this latter loss is unaffected by variations in the period of starvation within the range used here.

Exposure of the animals to antibiotics after transfer to culture medium gave evidence that loss can indeed occur during the growth period. Irradiated, stationary-phase paramecia were transferred either shortly after irradiation or 24 hr later to culture medium containing 1 mg per ml of streptomycin (Lilly) or 0.5 mg per ml of chloramphenicol (Pfizer). The animals were left in the antibiotic for 3 hr before transfer to culture medium without the antibiotic. The data are shown in Table 2. The antibiotics decrease the amount of mutation just as they do for exponentially

TABLE 2

EFFECT OF ANTIBIOTICS DURING SUBSEQUENT GROWTH ON MUTATION INDUCED IN STATIONARY-PHASE PARAMECIA

Postirradiation starvation before antibiotic treatment, days	Antibiotic treatment	$M \pm$ s.e.
0	−	1.01 ± 0.05
0	+	0.88 ± 0.05
1	−	0.88 ± 0.05
1	+	0.81 ± 0.05

growing paramecia. (The effect of chloramphenicol on paramecia has not been reported previously but is much the same as that of streptomycin.) There is a statistically significant effect for the group transferred immediately but not for that transferred after 24 hr. The difference is in the same direction, however; and the mean square for interaction between treatment and time is not significant. The effect at 24 hr would be expected to be small if a lower limit is being approached.

Discussion.—In our earlier papers[5–7] on postirradiation treatments, the hypothesis was advanced that (1) loss of premutational damage occurs up to the terminal event, (2) this event is some feature in the normal development for division, and (3) posttreatments decrease detectable mutation by allowing more time for loss. The previous data made this hypothesis quite attractive, but none of its special features were completely demonstrated. The present experiments give strong support to the first two assumptions and give some information about the third.

The second assumption was based on the observation that posttreatments, to be effective, had to be started before the middle of the interdivision interval. This moment was also marked by a sudden change in apparent "sensitivity" to mutation induction and has recently been shown to be the time at which DNA synthesis starts in the macronucleus.[13] The time of DNA synthesis in the micronucleus, the nucleus in which mutations are detected, has not yet been ascertained. The present studies show that the period in which modification can occur does not come to a sudden end when the paramecia are not growing and makes the previous conclusion that the terminal event is a normal feature of development for division still more probable.

The first assumption is also confirmed by the present data. The main evidence for this in the previous studies was the observation that the earlier in the interdivision interval the radiation was given, the less the mutation. This was interpreted to mean that the long intermediate period after early irradiation gave more time for loss of premutational damage, but a gradual increase during the interval in susceptibility to initial damage could not be excluded. The present data prove unambiguously that loss of premutational damage can occur during the intermediate period. This is shown, however, only for paramecia in the stationary phase, and final evidence for exponential growth will have to come from other experiments.

Suggestive evidence for an extension to exponential growth is provided by the present results, however. In the first place, the experiments with antibiotics show that irradiation of starved paramecia produces premutational damage that can be decreased by antibiotic treatment during subsequent growth. It seems probable that this is the same premutational damage that is lost during starvation since 1 day's starvation produces a very similar decrease in the amount of additional loss caused by either antibiotics or further starvation.

The second argument that supports loss during growth comes from a comparison of the maximum mutation produced by 4.5 kr to stationary and exponentially growing paramecia. Irradiation of the latter just before the presumptive terminal event gives a value for mutation of 1.7 *M*. Irradiation 2.5 hr earlier, at the beginning of the interdivision interval, gives 1.3 *M*. The maximum for irradiation in the stationary phase followed immediately by transfer to culture medium is 1.2 *M*. It is possible, of course, that starvation decreases the susceptibility to initial damage, but there is no a priori basis for predicting such an effect or its direction, and the physical state of the micronucleus, as far as it can be judged by microscopic observation, is not changed by starvation. The hypothesis of loss of premutational damage during growth predicts both the existence and direction of the effect since, even with immediate transfer to culture medium, a long intermediate period must intervene before the terminal event is reached.

The final feature of the original hypothesis that we wish to discuss is the assumption that decrease in mutation by postirradiation treatment is primarily the consequence of the increased time available for loss of premutational damage. The present data suggest, however, that the rate of loss of premutational damage is influenced by the metabolic state of the cell. The rate of loss during exponential growth can be estimated from the data in the previous paragraph to be about 0.4 *M* in 2.5 hr, on the assumption that the difference between irradiating early and late in the interdivision interval is entirely caused by the difference in time available for loss. The rate of loss in stationary-phase paramecia is approximated by the initial slope in Figure 1 and is obviously much lower than during exponential growth. This leads to the conclusion that loss is a metabolic process proceeding more rapidly in growing than in stationary cells. Thus the original hypothesis must be modified to take into account metabolic influences on the rate of loss. This does not alter the original conclusion that decrease in mutation is caused by increase in the time available for loss, provided agents such as streptomycin decrease the rate of loss. It would mean that the two effects, increase in time and decrease in rate of loss, are often balanced in such a way that the amount of mutation is decreased. These problems are under further investigation.

The problem of determining the rate of loss of premutational damage requires a little more discussion. Most postirradiation procedures must be assumed to influence both the rate of loss and the time available for it, unless there is evidence to the contrary. Thus the slope of the curve relating mutation to the duration of a posttreatment cannot, in general, be separated into the two component effects unless additional information is provided. In the case of stationary-phase paramecia, evidence has been presented that additional starvation does not affect the duration of the poststarvation interval (Table 1). Even then, the damage lost during this interval is probably a function of the damage remaining at its beginning; i.e., of the duration of the postirradiation starvation. The initial slope of the curve in Figure 1 should provide a good approximation, however, to the rate of loss of premutational damage during the stationary phase since only a small change in *M*, and so presumably in premutational damage, is caused by several hours of starvation. Discussion of measurement of the rate of loss for other organisms and by other methods in *Paramecium* will be deferred until a later publication.

The present experiments demonstrate that at least some forms of premutational

damage undergo loss during the intermediate period and lead to mutation only during the terminal event. Other processes, conversion to mutation during the intermediate period or increase in premutational damage with time, would lead to an increase instead of a decrease in mutation with increase in postirradiation starvation. Such processes might occur and be overbalanced by the loss process, and in this case, the lower limit in Figure 1 could be accounted for by an eventual balance between loss and gain. It is quite probable, however, that some mutation is included in the initial damage, and this alone could account for a lower limit. Consequently, there is no need at present to assume any form of premutational damage other than that subject to loss during the intermediate period and conversion to mutation during the terminal event.

Two main suggestions have been made about the influence of metabolism on the mutational process, in addition to its effects on the duration of the intermediate period. On the one hand, synthesis of macromolecules has been postulated[1, 12] to convert premutational damage to mutation; on the other, synthesis of such molecules has been thought[12, 14–17] to repair or eliminate radiation damage. The *Paramecium* results are more in accord with the latter view because there is no direct evidence for conversion to mutation before the terminal event, and loss of premutational damage seems to occur at a higher rate in rapidly growing than in stationary cells. Metabolic processes could also be involved in the conversion to mutation during the terminal event, but no evidence for this is yet available in *Paramecium*.

Summary.—Previous work with exponentially growing paramecia led to the hypothesis that part of the recessive lethal and other deleterious mutations induced by radiation in *Paramecium aurelia* comes from premutational damage that is subject to loss up to a terminal event, about midway through the interdivision interval. At this time the remaining damage leads to mutation. The present studies show that, during the stationary phase, loss of radiation-induced premutational damage continues until a lower limit, possibly mutation induced at the time of irradiation, is reached. The data unequivocally demonstrate loss of premutational damage; this phenomenon had previously been inferred on somewhat equivocal grounds. They also support the earlier conclusion that the terminal event is something in the normal development of growing cells since there is no evidence for such an event in nongrowing cells. It is suggested that loss of premutational damage is a metabolic process, possibly involving repair, since the rate of loss is much lower in the stationary phase than in exponential growth.

* Operated by Union Carbide Corporation for the United States Atomic Energy Commission

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*CHROMOSOME BREAKAGE PRODUCED BY TRITIUM-LABELED THYMIDINE IN TRADESCANTIA PALUDOSA**

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Communicated by Berwind P. Kaufmann, April 20, 1959

Introduction.—The original preparation of tritium-labeled thymidine by W. L. Hughes[1] and its application by Taylor, Woods and Hughes[2] in studies of chromosome duplication have led to wide-scale use of this labeled material in other areas of research. The fact that thymidine is a specific precursor of deoxyribonucleic acid (DNA) and that autoradiographs of very high resolution are obtainable using tritium have triggered most of these inquiries. Partial listings of the numerous investigations in which H^3-thymidine has been used are given by Cronkite *et al.*[3] and Hughes.[4]

Most of these studies have been concerned with the synthesis of DNA, the duplication, behavior and structure of chromosomes and the kinetics of cell populations, but relatively little research has been devoted to the possible biological effects of the internal soft beta radiations emitted by tritium. Although most workers have never doubted that some damage occurs, the extent of the damage has not been satisfactorily determined. Recently Plaut[5] has cautioned that use of H^3-thymidine in experiments may lead to erroneous conclusions because of the endogenous radiation. Painter, Drew, and Hughes[6] in a current paper have shown a marked inhibition of HeLa cell cultures grown on media containing 2.5 to 5.0 μc/ml of H^3-thymidine. Taylor[7] noted that fragments and interchromosomal exchanges were sometimes observed in squash preparations of *Bellevalia romana* roots that had been grown in nutrient solutions containing 2.5 to 5.0 μc/ml H^3-thymidine. In the present work chromosome fragmentation and mitotic inhibition are shown to occur in an organism treated with H^3-thymidine, and the degree of fragmentation will be related to amount of intranuclear irradiation measured by means of autoradiographs.

Materials and Methods.—Root tips of *Tradescantia paludosa* were used as experimental material. Plantlets were removed from the clonal parent (B2-2)[8] and the bases were placed in aerated Hoagland's solution under constant light (*c.* 400 ft. candles) and constant temperature (20° ± 1°C). After 72 hours most of the plantlets showed well developed roots.

Two procedures were used for labeling the nuclei. Some rooted plantlets were grown continuously in aerated nutrient solution containing 1 μc/ml H^3-thymidine

(0.36 C/mM supplied from Schwarz Laboratories, Mt. Vernon, New York) for 8 to 56 hours before fixing the root tips; others were grown in media containing 1 or 2 μc/ml for 4, 6, or 8 hours, washed thoroughly, transferred to isotope-free nutrient solution and fixed after 0 to 20 hours of further growth.

Roots were fixed in acetic-alcohol (1:3), hydrolyzed for 9 minutes in 1 *N* HCl at 60°C and stained by the Feulgen technique. Squashes were prepared in 45 per cent acetic acid and analyzed for fragments at anaphase. The squashes to be autoradiographed were frozen on dry ice, the cover slips removed and the slides placed immediately in 100 per cent ethanol. They were hydrated slowly and stripping film applied (AR 10 Kodak) according to the procedure outlined by Taylor.[9] The film was exposed for 24 hours and then developed. Activated silver grains over individual nuclei were counted.

Results and Discussion.—Mitotic figures in squashes from root tips grown in 1 μc/ml H^3-thymidine for 4 to 56 hours were scored for anaphase fragments. While aberrations were found in cells from roots in the labeling solution for 20 hours or more, it was soon apparent that there was a great variability of response of plantlets that had been grown in this solution for lengths of time over 8 hours; results from roots on a single plantlet seemed to show close agreement, but differences in frequencies of fragments between plantlets were sometimes rather great. These differences were thought to have arisen due to the variable number of roots on each plantlet and from the fact that a limited quantity of solution was available to each plantlet (10 ml); thus, the activity of those solutions with more roots would drop more rapidly than the average. However, if the data from several plantlets were grouped together, the results seemed to show a general trend. Through 16 hours few acentric fragments were noted, but from 20 to 24 hours there was a sharp increase in fragment frequency. There was then a drop to a relatively constant number of fragments which was maintained from 40 to 56 hours. These results are summarized in Table 1. The fact that the aberration frequency did not go

TABLE 1

THE EFFECT OF DURATION OF TREATMENT WITH 1 μc/ml H^3-THYMIDINE ON THE FREQUENCY OF ANAPHASE FRAGMENTS IN ROOT TIPS OF *Tradescantia paludosa*

Hours of Treatment	No. of Anaphases Analyzed	No. of Fragments	Fragments per 100 Cells
Control	1000	6	0.6
4	437	5	1.1
8	376	6	1.6
16	621	19	3.1
20	586	114	19.4
24	893	318	35.6
36	987	268	27.1
40	927	187	20.2
48	1625	307	18.9
56	980	200	20.4

higher after 24 hours could be attributed to a depletion of the H^3-thymidine in the nutrient solution. Scintillation counting for beta activity in the spent solutions in comparison to fresh isotope nutrient bore out this postulate. The presence of bridges, fragments, and micronuclei was similarly reported in root tip cells of *Allium cepa* grown in C^{14}-thymidine for extended lengths of time by McQuade, Friedkin, and Atchison.[10, 11]

In addition to an increase in fragment frequency, there was a drop in the mitotic index (cells in mitosis/total cells × 100) in roots that had been immersed in solutions of H^3-thymidine for over 8 hours. Normally the index was about 15 per cent in control roots; in some treated roots the index had dropped to 3 per cent.

In those preparations which had been scored previously for fragments and to which stripping film had been applied, it was possible to study fragment frequency as a function of grain density or extent of incorporation of H^3 into the chromosome. The number of grains over prophase, metaphase, anaphase, and telophase nuclei of roots grown from 24 to 56 hours in H^3-thymidine solutions was determined and the data were plotted against the number of fragments per 100 anaphase cells. The results are shown in Figure 1. Each point represents the combined data from several roots taken from one plantlet. The squashes selected for grain counting were purposely chosen for their high fragmentation in order that the percentage

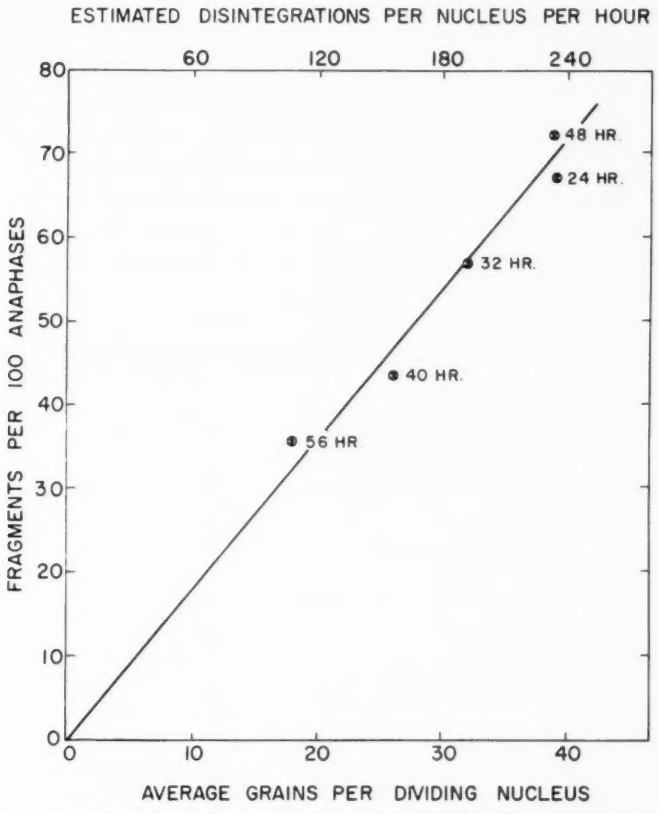

FIG. 1.—Increase in chromosome fragmentation in *Tradescantia paludosa* exposed to 1 μc/ml H^3-thymidine as a function of autoradiographic grain number over dividing nuclei. 200 or more cells were scored for each point. Disintegrations per nucleus calculated on basis of 1 grain per 143 disintegrations.

error in scoring so few cells would be at a minimum; thus, the average number of fragments per 100 cells found in Table 1 is always lower than those shown in Figure 1. The curve shows a roughly linear increase of grain count with degree of fragmentation. Within the limits covered by the observations the degree of fragmentation is proportional to the average amount of thymidine incorporated.

Delay of mitosis by intranuclear irradiation is indicated by the observation that nuclei that showed very high grain counts, i.e., 80 grains or more, were seldom found in division. Although nuclei were frequently observed with over 100 grains, they were almost without exception in interphase. For example, of 2145 interphases with more than 4 grains per nucleus, 11.5 per cent showed more than 80 grains over the nuclei; on the other hand, of 1512 labeled cells in division only 0.3 per cent showed over 80 grains. The difference is even greater than these figures indicate since the efficiency of the autoradiograph for nuclei in different stages must be taken into account. The average grain counts were about equal over all of the mitotic stages, i.e., prophase, metaphase, anaphase and telophase; furthermore, it was determined that cells in mitosis showed about one third more grains than cells in interphase. Thus, a grain count of 80 during mitosis would correspond to about 60 during interphase. Of the 2145 interphases examined, 15.9 per cent had more than 60 grains over the nuclei.

Because a number of workers have utilized roots that had been grown from 6 to 10 hours in H^3-thymidine (*c.* 2 μc/ml), it was deemed of interest to follow this same procedure and to look for fragments after further growth in isotope-free nutrient solutions for periods up to 24 hours. The results are reported in Table 2. The shorter periods of exposure of 4 to 8 hours in this experiment versus the longer

TABLE 2

CHROMOSOME FRAGMENTATION IN *Tradescantia paludosa* ROOT TIPS AS INDUCED BY EXPOSURE TIMES OF 4, 6 AND 8 HOURS IN 1 AND 2 μc/ml OF H^3-THYMIDINE

	1 μc/ml			2 μc/ml		
Collection Hour*	No. of Anaphases Analyzed	No. of Fragments	Frag./100 Cells	No. of Anaphases Analyzed	No. of Fragments	Frag./100 Cells
			4 hour exposure			
4	321	4	1.2	620	8	1.3
8	309	8	2.6	279	8	2.9
12	175	12	6.8	386	52	13.5
16	102	15	14.7	133	23	17.3
20	301	8	2.7	343	25	7.3
24	213	11	5.2	196	4	2.0
			6 hour exposure			
6	125	1	0.8	437	5	1.1
8	159	4	2.5	150	9	6.0
12	147	14	9.5	199	50	25.1
16	266	42	15.8	347	84	24.2
20	267	5	1.9	482	92	19.1
24	104	5	4.8	625	25	4.0
			8 hour exposure			
8	158	5	3.2	62	0	0.0
12	119	20	16.9	191	21	11.0
16	179	18	10.0	224	71	31.7
20	126	11	8.7	389	117	30.1
24	1060	25	2.4	589	81	13.7

* Zero hour is the time at which the plantlets were initially placed in the isotope solution. At 4, 6, and 8 hours the plantlets were changed to isotope-free solution and grown for various lengths of time up to the twenty-fourth hour.

periods of up to 56 hours in the other experiment minimized the interplantlet variation. Few fragments were found upon immediate examination of the root tips that had been immersed in the H^3-thymidine solution for 4 to 8 hours. However, a significant increase in the number of fragments was apparent when the root tips were allowed to continue division in isotope-free solution. For example, cells from roots that had been grown for 4 hours in 2 μc/ml H^3-thymidine, and then transferred to a nonisotopic solution for further growth showed a statistically significant increase in fragmentation at the twelfth and sixteenth hours from time of initial immersion into the isotope solution, i.e., 13.5 and 17.3 fragments per 100 cells respectively. Generally, there was a decrease in fragment frequency at the twentieth hour although data from roots that were treated for 6 and 8 hours in 2 μc/ml showed a slower decline than the data from those treated for a shorter time or with a lower isotope concentration, i.e., the fragment values were 19.1 and 30.1 per 100 cells for 6 and 8 hour exposures respectively; other values for the twentieth hour were less than half the above-mentioned figures.

From some other experiments that will be reported elsewhere it is known that the mitotic cycle in *Tradescantia paludosa* may be divided into the following average times and stages in relation to DNA synthesis:

(1) 4 hours, the period preceding DNA synthesis during interphase (G_1).
(2) 10 hours, DNA synthesis (S).
(3) 3 hours, non-DNA synthetic period before mitosis (G_2).
(4) 3 hours, mitosis (M).

These figures are similar to those reported for *Vicia faba* by Howard and Pelc.[12] Considering this time scale a simple estimate can be made of the length of time it will take for cells that had been in DNA synthesis during the total labeling period to reach anaphase, providing certain assumptions are made: namely, that the cells are all proceeding through the mitotic cycle at the same rate, that DNA synthesis is equally rapid during most of the synthetic period and that the precursor pool comes into equilibrium with the labeling solution very quickly. For those roots that had been grown in H^3-thymidine for 4 hours and then transferred to isotope-free nutrient, cells that had the maximum incorporation of this material would appear at anaphase between 9 and 15 hours; for the 6 hour sample, the most highly labeled anaphases would appear at 11 to 15 hours while in the 8 hour case they would be seen at 13 to 15 hours.

It is a reasonable expectation for those cells that absorbed the largest amount of H^3-thymidine to contain the greatest number of fragments at anaphase. The fragment data presented in Table 2 tend to confirm this hypothesis for there was an increase in fragmentation for the twelfth to the sixteenth hour and in some with the longer treatment the twentieth hour was involved. Even though at 24 hours most of the anaphases possessed few fragments, micronuclei were found to be rather common if the interphase nuclei were examined.

The findings presented above are of particular interest, since a number of researchers have utilized rather high concentrations (± 2 μc/ml) of H^3-thymidine for 6 to 10 or more hours. Taylor,[7] in particular, reported sister-chromatid exchanges at the second division after labeling *Bellevalia romana* root tips for 6 to 10 hours with 1.6 to 2.5 μc/ml of solution. He mentioned that these exchanges were possibly caused

by the endogenous radiation, but in a later publication[13] he seemed to think that their frequency was unaffected by the internal radiation. From the data herein presented it is evident that chromosome breakage is frequent; it is hardly necessary to conjecture that subchromatid damage would also be relatively common at comparable levels of activity and exposure times.

The abrupt rise in the number of chromosome fragments in the roots receiving continuous treatment (Table 1) occurred between 16 and 20 hours. In roots treated for a short time with the labeled precursor followed by growth in a nonlabeled medium (Table 2), the time of rise occurred generally much sooner, i.e., 12 to 16 hours. This delay in the rise of the aberration frequency may have been due to a cumulative radiation effect on the mitotic cycle. It is a well-known fact that one of the characteristic features of radiation damage is a delay in the onset of mitosis.

An estimation of the dose that each nucleus received from the endogenous decay of the tritium would be of interest but there are several obstacles that present themselves in calculating a correct dose. One of the foremost is the fact that the H^3 is confined almost entirely to the thymine of the DNA in the chromosomes. This could result in a nonrandom distribution of the tritium within the nucleus and also a nonrandom distribution of the beta rays.

One of the possible methods of estimating the dose is by a comparison of the fragment frequency with data from experiments with exogenous radiation. Mikaelsen[14,15] grew plantlets of the same clone of *Tradescantia paludosa* utilized here and exposed the roots for 48 hours to continuous gamma radiation from a Co^{60} source at an intensity of about 0.9 rad per hour. At the end of the treatment the average number of fragments per 100 cells at anaphase was reported as 22.1. Data given by Gray[16] indicated that β rays of the energy emitted by H^3 are about three times as effective per absorbed dose as γ rays in producing chromosome breaks. From Figure 1 roots exposed to H^3-thymidine for 24 hours show a fragment frequency of 67.5 per 100 cells. Although no direct comparison of these findings may be made to Mikaelsen's data, the dose rate delivered to the nuclei by the H^3 in this case is probably no more than five times that of the gamma intensity, i.e., less than 4.5 rads per hour.

Theoretically a rather accurate assay of the dose delivered to each nucleus could be made on the basis of grain counts over the nuclei. Hughes and co-workers[17] have made some estimates of the efficiency of grain counts in assaying the total number of disintegrations per nucleus. They judge that in a tissue section about one grain is formed per 50 disintegrations over nuclei that have been cut in two. The real value is probably somewhere between 10 and 100 disintegrations per activated grain. Although the actual steps will be omitted here, it may be calculated that an average grain number of 36.6 over dividing nuclei in a squash preparation produced by a film exposure of 24 hours would deliver to the nuclei between 5.2 and 52 rads per hour. Expressing it another way, roughly one anaphase fragment would result from 64.6 to 646 disintegrations per hour per 100 nuclei at the time of fixation; this estimate assumes a direct functional relationship between fragment frequency and autoradiographic grain number. In Figure 1, where grain count is plotted against fragment frequency per 100 cells, a grain count of 36.6 can be correlated with an induced fragment frequency of 67.5 per 100 cells. The estimates of dosage based on two different criteria can now be compared, i.e., 5.2—52 rads/hour versus <4.5 rads/hour.

Certainly the lowermost calculation based on grain count is the most compatible with the estimate taken from Mikaelsen's data. But, until more accurate measurements of tritiated substances in producing autoradiographs are available, we shall probably be unable to make more accurate estimations of the actual dose received.

Summary.—The fragmentation of chromosomes in the root tips of *Tradescantia paludosa* as produced by tritium-labeled thymidine was measured. As many as 31 fragments per 100 cells occurred in roots grown for 4 to 8 hours in 1 or 2 μc/ml H^3-thymidine and then transferred to isotope-free solutions for further growth before collection. The highest fragment frequency could be correlated with the period during which the maximum amount of H^3-thymidine was incorporated into the chromosomes. Up to 72 fragments per 100 cells at anaphase were demonstrated after continuous exposure of root tips to 1 μc/ml H^3-thymidine for times up to 56 hours.

The mitotic index dropped sharply in roots that had been exposed to 1 μc/ml of H^3-thymidine for periods over 8 hours. Cells that absorb large quantities of H^3-thymidine as indicated by autoradiographs are seemingly disturbed in their development and are delayed or prevented from entering mitosis. Autoradiographs of roots that had been exposed to 1 μc/ml H^3-thymidine for 24 to 56 hours showed that the average grain count over dividing nuclei increased as the fragmentation appearing at anaphase increased.

Approximations of the dose delivered to the nuclei by the endogenous radiation from the tritium were made by two different methods; however, the estimates showed poor agreement. Confident calculations of the dose probably await the determinations of the efficiency of the β rays in producing an autoradiograph.

These findings would indicate that investigators utilizing tritiated substances should interpret their results with caution for the endogenous radiation delivered to a cell may be great enough to cause considerable chromosome breakage and presumably other forms of genetic damage as well as significant changes in cellular physiology.

The author gratefully acknowledges the advice of Dr. A. H. Sparrow during the course of the work and discussions with Drs. P. S. Woods, D. Steffensen, H. Quastler, and Miss V. Pond during the preparation of the manuscript.

* This investigation was carried out while the author was a Public Health Service Research Fellow of the National Cancer Institute (CF-8465) at the Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

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*ENZYME COMPLEMENTATION IN VITRO BETWEEN ADENYLOSUCCINASELESS MUTANTS OF NEUROSPORA CRASSA**

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Communicated by Edmund W. Sinnott, April 24, 1959

Previous studies[1] have shown that certain mutant strains of *Neurospora crassa* lacking detectable adenylosuccinase activity, and hence unable to grow in the absence of exogenous adenine, complement one another in certain combinations when grown as heterocaryons. Such complementation results in adenine-independent growth, and certain of the resulting heterocaryons possess a maximum of 25 per cent of wild-type adenylosuccinase activity. The pattern of complementation has been depicted as a complementation map of the *ad-4* locus and suggests a structural correlation between gene and gene product.[2–4] These results led to the hypothesis that complementation involves a cytoplasmic exchange mechanism operating at either the RNA or polypeptide level. The work of Singer and Itano,[5] demonstrating the formation of hybrid protein *in vitro*, lends further support to the suggestion that exchange at the polypeptide level could provide a mechanism for complementation. In addition, such a mechanism could also explain the restoration of enzyme activity in heterocaryons to a maximum level not exceeding 25 per cent of wild type. Other recent studies[6–9] suggest related types of exchange mechanisms that could also yield hybrid protein or protein complexes.

The above hypothesis has received additional support with the demonstration of *in vitro* complementation. By mixing mycelial homogenates from certain pairs of complementing adenylosuccinaseless *Neurospora* mutants grown separately, adenylosuccinase activity as high as 20 per cent of that obtained from *in vivo* complementation has been recovered. The same procedure performed with complementing mutants, singly, or with mixtures of non-complementing mutants, has failed to restore detectable enzyme activity. Earlier attempts[1] to detect enzyme activity in mixtures of extracts from complementing *ad-4* mutants gave negative results.

Materials and Methods.—Data on the origin and other characteristics of the *ad-4* mutants employed in these studies have been presented.[4]

The following general procedure has been found to be the most effective in demonstrating *in vitro* complementation in mixing experiments. Mutant cultures are grown separately for 3–4 days at 25°C on Fries minimal medium containing 100

μg/ml of adenine sulfate. Mycelial pads are removed, washed in chilled distilled water, pressed dry on absorbent paper and quick frozen. Frozen samples are mixed, and the cells are disrupted in a Hughes Press at 5000 psi. The frozen powder from the mixed samples is taken from the press and diluted to a liquid paste condition with $M/20$ tris HCl buffer containing 10^{-3} M reduced glutathione (pH 7.0) and placed on a shaker for 10–15 min or until the frozen extract thaws. The pH is adjusted to approximately 8.0 and the mixture diluted further (usually a two-fold dilution); the mixture is agitated for another hour at 4°C. Cell debris is removed by centrifugation at about 50,000 $\times$ g for 30 min. The supernatant solution is further purified by column chromatography on DEAE cellulose.[10] The column purification is not essential to the recovery of enzyme activity, but some of the ultraviolet-absorbing material that would otherwise interfere with the assay is removed. The assay used is a measure of decrease in absorption at 280 mμ as the reaction proceeds from adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP).[1]

The assumption that the decrease in absorption observed at 280 mμ is a measure of the rate of conversion of AMP-S to AMP is based on spectral scans during the reaction. These scans show a change in maximum absorption from 267 mμ (AMP-S peak) to near 260 mμ (AMP peak). This is the same spectral change observed when the wild-type enzyme is used, but a change that does not occur when single mutant extracts are used. A maximum change in absorption occurs at 280 mμ when either wild-type enzyme or enzyme recovered as a result of *in vitro* or *in vivo* complementation is reacted with the AMP-S substrate. The rate of change in absorption is measured on a recording spectrophotometer and is linear for more than 30 min; thus quantitative determinations can be quite precise.

Results and Discussion.—The results of various mixing experiments performed to date are presented in Table 1. At this stage of the investigation, it appears premature to draw many conclusions about the *in vitro* restoration of enzyme activity except that it is accomplished by the procedure described. Other attempts using variations of this procedure and other extraction procedures have failed to restore enzyme activity. Negative results were obtained whenever mycelia were lyophilized before mixing in the extraction buffer; similarly, when the mycelia were ruptured by grinding in sand with mortar and pestle, no enzyme activity was recovered. From the mixing experiments that have been performed, it appears that the process through which restoration of enzyme activity is accomplished is short-lived under the particular experimental conditions used. Mixing must take place within 5–10 min after the extracts have thawed or no enzyme activity is restored. It does not seem likely that the procedure used exerts its influence through the activation of an inactive enzyme since mutants treated alone exhibit no enzyme activity.

The presence of 10^{-3} M reduced glutathione during extraction appears to improve the recovery of enzyme activity, and there is some indication that ATP enhances the recovery, but highly refined quantitative measurements are not possible at this time.

Additional data have also been obtained from further studies involving *in vivo* restoration of enzyme activity. These data support earlier findings[4] of a correlation between complementation map distance and the levels of adenylosuccinase

TABLE 1

ADENYLOSUCCINASE ACTIVITY RESTORED THROUGH *IN VITRO* COMPLEMENTATION

Mutants	Additives During Extraction	Specific Activity (ΔA/mg. P/min/ml)	% of *in Vivo* Activity
F4 and F39 (heterocaryon)	None (no significant variation with additives present)	1.90	100.0
F4 and F39 (mixture)	None	0.19	10.0
		0.20	10.6
	ATP (Ave. of 3 experiments)	0.30	15.8
	ATP + GSH + NaF	0.31	16.4
		0.28	14.7
F4 and F48 (heterocaryon)	None (no significant variation with additives present)	1.60	100.0
F4 and F48 (mixture)	None	0.15	9.4
	GSH	0.33	20.6
F39 and F31 (heterocaryon)	None	1.17	100.0
F39 and F31 (mixture)	None	0.20	17.1
F4 and F31 (heterocaryon)	None	0.80	100.0
F4 and F31 (mixture)	None	0.04	5.0
F4	All variations	0.00	0.0
F39	All variations	0.00	0.0
F48	All variations	0.00	0.0
F31	All variations	0.00	0.0
F4 and F2* (mixture)	GSH	0.00	0.0
F39 and F2* (mixture)	GSH	0.00	0.0

* Non-complementing combination *in vivo*.

activity found in interallelic heterocaryons. The results plotted in Figure 1 indicate a steady increase of enzyme activity with increasing map distance up to a separation of about 4 cistrons, at which point no further significant increase in activity is observed. According to the hypothesis,[4] if the sites of damage in the two different defective gene products produced by the two complementing mutant nuclei are sufficiently widely separated such that random exchange can occur, then such a heterocaryon should yield 25 per cent of wild-type enzyme activity. This theoretical maximum of 25 per cent was in fact the maximum adenylosuccinase yield observed. However, an attempt to establish the precise nature of this correlation between map distance and enzyme activity does not yet appear justified in view of the limited data and the variability that exists.

Obvious irregularities in the plot of map distance vs. enzyme activity are limited to one or two cases. The one adjacent cistron combination that yields 10 per cent of wild-type enzyme activity may be such an exception. However, this discrepancy may be more apparent than real. For example, it is not yet certain that all of the cistrons have been located at the *ad-4* locus. If a cistron not yet detected exists between the two presumably adjacent cistrons, the 10 per cent value observed would fit into the plot of Figure 1 very well. This particular heterocaryon combination presents an additional complication since one of the mutant components (F23) is leaky to the extent of almost one per cent of wild-type adenylosuccinase activity even though it is unable to grow on minimal medium. The leakiness may contribute to the unexpectedly high 10 per cent recovery. The only other adjacent cistron combination studied (F12 × F14) possessed less than one per cent of wild-type enzyme activity. Because of poor growth in other adjacent cistron combinations, assays for enzyme activity were not possible. Other variables involved in making specific activity determinations are either inherent in the assay procedure or are brought about by unknowns present in the crude extract.

Heterocaryons have also been made between F12 reversions that possess only

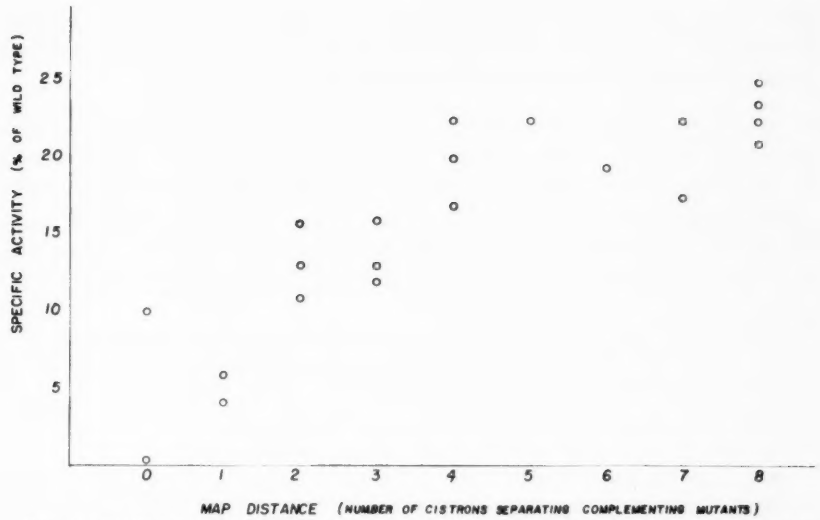

FIG. 1.—Relationship between complementation map distance and adenylosuccinase activity in heterocaryons. Each point plotted represents a heterocaryon composed of different pairwise combinations of adenylosuccinaseless mutants. Many of these figures are averages from several assays of separately grown cultures of the heterocaryons. The specific activity is a measure of the $\Delta A/28$ mg dry wt/20 min/0.77 ml. expressed as a per cent of wild-type activity.

3 per cent of wild-type enzyme activity and mutants that complement F12 and are widely separated from F12 on the complementation map. Since there is now evidence[11] indicating qualitative differences between the enzymes from these reversions and the wild-type enzyme, the assumption can be made that the low level of enzyme activity in the reversions is not due to any quantitative change in the amount of protein synthesized but only to such qualitative changes.[12] On this basis, the hypothesis of random exchange for the wide separation involved predicts a maximum of 25.75 per cent of wild-type enzyme activity. The actual figures obtained from these heterocaryons were 24.3, 24.0, 22.0, and 22.4 per cent of wild-type adenylosuccinase activity.

Until more is understood about *in vitro* complementation, definitive statements concerning the mechanism involved cannot be made. Nevertheless, the present system appears to provide an excellent opportunity for studying this problem.

Summary.—*In vitro* complementation between homogenates from *Neurospora* adenylosuccinaseless mutants resulting in restored adenylosuccinase activity is described. As much as 20 per cent of the activity recovered from *in vivo* complementation in heterocaryons has been restored by the *in vitro* mixing procedure. If the homogenates from two complementing mutants are not mixed immediately after the mycelia have been disrupted, enzyme activity is not recovered. No enzyme activity has been detected when homogenates from non-complementing mutants are mixed. *In vivo* complementation results in the restoration of enzyme activity to a maximum level not exceeding 25 per cent of wild-type. There is a direct correlation between enzyme activity recovered from interallelic heterocaryons and distance between the mutant components on the complementation

map. These results support the hypothesis previously proposed that, in general, interallelic complementation in enzyme formation involves a cytoplasmic exchange mechanism resulting in the formation of hybrid protein.

The author is greatly indebted to Dr. Norman H. Giles for his guidance throughout this investigation. The author is also grateful to Drs. C. W. H. Partridge, S. J. Singer and V. W. Woodward for helpful suggestions concerning this manuscript.

* This research was performed during the tenure of a pre-doctoral fellowship, MF-7405, of the National Institute of Mental Health, Public Health Service, and was supported in part by a research contract with the Atomic Energy Commission, AT (30-1)-872, administered by Norman H. Giles. This paper is a part of a dissertation presented for the degree of Doctor of Philosophy in Yale University.

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LOWER BOUNDS FOR EIGENVALUES WITH APPLICATION TO THE HELIUM ATOM

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Communicated by Eugene P. Wigner, April 8, 1959

In the present paper we shall outline a method for finding lower bounds to the discrete eigenvalues of a self-adjoint operator; upper bounds are always comparatively easy to obtain by the Rayleigh-Ritz technique. Our procedure for lower bounds goes back to the method of A. Weinstein,[1] which introduces an explicitly solvable *base problem* with lower eigenvalues. A. Weinstein links the base problem to the given problem by a sequence of *intermediate problems* which can be solved in terms of the base problem and which improve the lower bounds. In his work on plates these problems are obtained by changing the boundary conditions. By combining his lower bounds with the upper bounds given by the Rayleigh-Ritz method he obtained an accuracy of up to 0.18 per cent.

Later Aronszajn[2] emphasized that a base problem can be obtained by a change of the operator and outlined the construction of the intermediate problems. In this paper we simplify Aronszajn's method by constructing intermediate problems in such a way that in order to solve them in terms of the base problem one need only solve an algebraic equation instead of a transcendental equation. As an applica-

tion we compute energy levels of the helium atom, for which there have been no rigorous numerical results on lower bounds.

Let A be a self-adjoint operator which is bounded below in the separable Hilbert space $\mathfrak{H}$ with scalar product (u, v). We denote the domain of A by D. We assume that A has the decomposition

$$A = \bar{A} + A', \tag{1}$$

where $\bar{A}$ is a self-adjoint operator with domain D for which the eigenvalue problem is completely solvable, and A' is a symmetric operator with domain $D' \supset D$ which satisfies $(A'u, u) > 0$ for $u \neq 0$. We call the eigenvalue problem for $\bar{A}$ the base problem.

For simplicity we assume that the lowest parts of the spectra of both $\bar{A}$ and A are discrete and denumerably infinite, with finite multiplicities. We then have, by the maximum-minimum principle for eigenvalues, the fundamental inequalities

$$\bar{\lambda}_i \leq \lambda_i \qquad (i = 1, 2, \ldots) \tag{2}$$

where $\bar{\lambda}_i$ and λ_i are the ordered eigenvalues of $\bar{A}$ and A respectively. We now introduce a sequence of problems with eigenvalues intermediate between those of $\bar{A}$ and A. The kth eigenvalue problem of this sequence is that of the operator

$$A^{(k)} = \bar{A} + A'P^{(k)}. \tag{3}$$

Here $P^{(k)}$ is the projection on a k-dimensional subspace $[\pi_1, \ldots, \pi_k]$ of the Hilbert space $\mathfrak{H}'$, where $\mathfrak{H}'$ is formed by completing D' in the norm $(A'u, u)^{1/2}$. It follows that $(A'P^{(k)}u, u) \leq (A'P^{(k+t)}u, u) \leq (A'u, u)$, where $P^{(k+t)}$ is the projection on a subspace of $\mathfrak{H}'$ which contains t additional basis vectors. The ordered discrete eigenvalues $\lambda_i^{(k)}$ of $A^{(k)}$ satisfy

$$\bar{\lambda}_i \leq \lambda_i^{(k)} \leq \lambda_i^{(k+t)} \leq \lambda_i \qquad (i, k, t = 1, 2, 3, \ldots). \tag{4}$$

To obtain significant results we choose

$$\pi_i = (A')^{-1}\bar{u}_i \qquad (i = 1, \ldots, k) \tag{5}$$

where $\bar{u}_i$ is the normalized eigenvector of $\bar{A}$ corresponding to $\bar{\lambda}_i$; we require that these π_i's exist. Then we have the following results.

The operator $A^{(k)}$ is self-adjoint and its spectrum is given by the rule:

(*a*) k of the eigenvalues are the roots of

$$0 = \det[(\bar{\lambda}_i - \lambda)\delta_{ij} + b_{ij}] \qquad (i, j = 1, \ldots, k) \tag{6}$$

where δ_{ij} is the Kronecker delta and b_{ij} is the element of the matrix inverse to that with elements $((A')^{-1}\bar{u}_i, \bar{u}_j)$.

(*b*) All other discrete eigenvalues are those $\bar{\lambda}_t$'s of the base problem whose corresponding eigenvectors $\bar{u}_t$ have not been used in forming any of the π_i's.

(*c*) The continuous spectrum is identical with that of the base problem.

In the application of this method to the Hamiltonian operator of the helium atom we neglect nuclear motion, relativistic effects, and the influence of spin. The self-adjoint Hamiltonian[3] in the Hilbert space $\mathfrak{H}$ of quadratically integrable functions is then

$$A = \frac{1}{2}\mathbf{p}_1^2 + \frac{1}{2}\mathbf{p}_2^2 - \frac{2}{r_1} - \frac{2}{r_2} + \frac{1}{r_{12}} \tag{7}$$

in atomic units. Here $\mathbf{p}_1$ and $\mathbf{p}_2$ are the momentum operators of the two electrons, $\mathbf{r}_1$ and $\mathbf{r}_2$ are their position vectors with the nucleus as origin, and r_{12} is their separation distance. The domain D of A is the set of all functions $f(\mathbf{r}_1, \mathbf{r}_2)$ whose representations $F(\mathbf{p}_1, \mathbf{p}_2)$ in momentum space satisfy the conditions

$$\int |F(\mathbf{p}_1, \mathbf{p}_2)|^2 d\mathbf{p}_1 d\mathbf{p}_2 < \infty, \int \mathbf{p}_i^4 |F(\mathbf{p}_1, \mathbf{p}_2)|^2 d\mathbf{p}_1 d\mathbf{p}_2 < \infty \qquad (i = 1, 2). \tag{8}$$

Kato[3] has shown that the lower part of the spectrum of A consists of a denumerable infinity of discrete eigenvalues.

If the electron interaction term $1/r_{12}$ is neglected the eigenvalue problem for the resulting Hamiltonian $\bar{A}$, also self-adjoint in D, has the discrete eigenvalues

$$-2\left(\frac{1}{n_1^2} + \frac{1}{n_2^2}\right), \qquad (n_1, n_2 = 1, 2, \ldots) \tag{9}$$

with multiplicities $n_1^2 n_2^2$, and corresponding eigenvectors

$$\psi_{n_1\iota_1 m_1}(\mathbf{r}_1)\psi_{n_2\iota_2 m_2}(\mathbf{r}_2), \ (n_i = 1, 2, \ldots; \iota_i = 0, \ldots, n_i - 1; m_i = 0, \pm 1, \ldots, \pm\iota_i) \qquad \text{for } i = 1, 2 \tag{10}$$

where the $\psi_{n\iota m}$'s are hydrogenic wave functions. There is a continuous spectrum, overlapping the discrete spectrum, which begins at -2 and extends to infinity.

We restrict ourselves to S-states of para-helium with the eigenvalues $E(1^1S)$, $E(2^1S), \ldots$. For these states the eigenfunctions of A depend only upon r_1, r_2, and r_{12} and are symmetric in the spatial coordinates of the two electrons. Since $(A')^{-1} = r_{12}$ implies $\pi_i = r_{12}\bar{u}_i$, the π_i's are given as

$$\pi_{n_1 n_2 \iota} = N r_{12}[R_{n_1\iota}(r_1)R_{n_2\iota}(r_2) + R_{n_1\iota}(r_2)R_{n_2\iota}(r_1)]P_\iota(\cos\theta_{12}), \ (n_1, n_2 = 1, 2, \ldots; \iota = 0, 1, \ldots, n^* - 1) \text{ for } n^* = \min(n_1, n_2) \tag{11}$$

where the $R_{n\iota}$'s are the normalized radial wave functions, $P_\iota(x)$ is the ιth normalized Legendre polynomial, and θ_{12} is the angle between $\mathbf{r}_1$ and $\mathbf{r}_2$. Here N is chosen so that the $\bar{u}_i$'s are normalized.

We have solved the third intermediate problem for the operator in (3) with a projection on the subspace spanned by π_{110}, π_{120}, and π_{130}. We order the three roots of equation (6) with those eigenvalues which persist from the base problem. While the base problem gives the inequalities $-4.0 \leq E(1^1S)$ and $-2.5 \leq E(2^1S)$, our intermediate problem yields the improved inequalities $-3.063_7 \leq E(1^1S)$ and $-2.165_8 \leq E(2^1S)$. Using the Rayleigh-Ritz upper bounds as determined by Kinoshita[4] and Coolidge and James,[5] we have

$$-3.063_7 \leq E(1^1S) \leq -2.9037225, \ -2.165_8 \leq E(2^1S) \leq -2.1458. \tag{12}$$

In the present example a numerical improvement to the lower bound for $E(1^1s)$ can be made by combining the results with Temple's method,[6] which is closely related to the work of Kryloff and Bogoliubov,[7] Kohn,[8] Kato,[9] D. H. Weinstein,[10] and Mäehly.[11] Temple's formula states that for arbitrary normalized $\psi \epsilon D$

$$E(1^1S) \geq (\psi, A\psi) - \frac{(A\psi, A\psi) - (\psi, A\psi)^2}{E^* - (\psi, A\psi)} \tag{13}$$

where E^* is a lower bound to $E(2^1S)$. This formula was previously applied by Kinoshita,[4] but he used the experimental value of $E(2^1S)$ for E^*. In our procedure we use the lower bound in (12) for E^* and the Kinoshita 39-parameter trial function for ψ to obtain the rigorous inequalities

$$-2.9038777 \leq E(1^1S) \leq -2.9037225. \tag{14}$$

The author wishes to express his gratitude to L. E. Payne and H. F. Weinberger for their kind interest. A more detailed presentation of this work will be submitted for publication to the *Journal of Mathematics and Mechanics*.

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FUNCTIONAL EQUATIONS AND THETA-FUNCTIONS—I

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Communicated by H. S. Vandiver, April 15, 1959

Introduction.—Consider the four theta-functions

$$\begin{aligned}
\theta_1(z) &= 2q^{1/4}\sin z - 2q^{9/4}\sin 3z + \ldots, \\
\theta_2(z) &= 2q^{1/4}\cos z + 2q^{9/4}\cos 3z + \ldots, \\
\theta_3(z) &= 1 + 2q\cos 2z + 2q^4\cos 4z + \ldots, \\
\theta_4(z) &= 1 - 2q\cos 2z + 2q^4\cos 4z - \ldots,
\end{aligned} \tag{1}$$

where $q = e^{\pi it}$ and $Im(t) > 0$. We are following the notation used in Whittaker and Watson.[1]

These functions are united by the fact that they satisfy the functional equations

$$\begin{aligned}
u(z + \pi) &= (\pm)\, u(z), \\
u(z + \pi t) &= (\pm)q^{-1}e^{-2iz}u(z),
\end{aligned} \tag{2}$$

taking all possible combinations of the $\pm$. In addition to these easily derived results, more recondite relations hold, such as

$$\theta_3(z + y)\theta_3(z - y)\theta_3^2(0) = \theta_3^2(y)\theta_3^2(z) - \theta_1^2(y)\theta_1^2(z) \tag{3}$$

and

$$\theta_3(z, t) = (-it)^{-1/2}e^{z^2/\pi it}\theta_3(z/t, -1/t) \tag{4}$$

the famous theta-function transformation of Poisson and Jacobi.[2]

These results, as well as practically all such results, were derived by Jacobi in his famous work, using purely algebraic arguments. The relation in (4) can be obtained from the theory of the heat equation, a method which is capable of yielding many further results, and can also be derived as an application of the Poisson summation formula, another method which yields extensive generalizations.

The customary proof of (3), and many other relations of this type, is by means of the theory of functions of a complex variable, specifically Liouville's theorem. Both methods available for the one-dimensional case, the algebraic and the function-theoretic, fail when we turn to the derivation of analogous results for higher dimensional theta-functions.

We wish here to present a new method, quite simple in nature, which can be used to treat both the one-dimensional and multi-dimensional case in a uniform manner. In this note we shall sketch the application to the one-dimensional case.

Difference Equations.—The basic idea is the observation that not only do the functions lead to the functional equations of (1.2), but the functional equations determine the theta-functions, to within a quantity independent of z. Consequently, it should be possible to derive all the properties of theta-functions from these functional equations, without recourse to the theory of functions of a complex variable.

If we consider an equation set of the form

$$\begin{aligned} v(z + \pi) &= v(z), \\ v(z + \pi t) &= be^{-2niz}v(z), \end{aligned} \tag{1}$$

where b is independent of z, and greater than one in absolute value, and n is one of the integers 1, 2, . . ., we can assert that there are precisely n linearly independent continuous solutions of period π. Furthermore, every other solution in a linear combination of these solutions.

Squaring the equations of (1.2), we immediately obtained the well-known result[3] that there exists a linear relation connecting the squares of any three of the four theta-functions of (1.1). Similarly, since $\theta_i(z + y)\theta_i(z - y)$, for $i = 1, 2, 3, 4$, satisfies these squared equations, we see the genesis of (1.3).

The relation of (1.4) is obtained, up to the determination of the factor $(-it)^{-1/2}$ from the uniqueness theorem for the case $n = 1$. The factor may be found by looking at the asymptotic behavior of the functions as t approaches 0.

Discussion.—In subsequent papers we will indicate how these techniques may be applied to the higher dimensional theta-functions, with particular reference to those of Hecke and Siegel. Furthermore, we will show how these methods can be applied to yield the well-known series expansions for the logarithmic derivatives, $\theta_i'(z)/\theta_i(z)$.

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*GENERALIZED CONJUGATE AND ANALYTIC FUNCTIONS WITHOUT EXPANSIONS**

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Communicated April 20, 1959

On a Lebesgue measure space with measure element $d\sigma$, and total measure finite or infinite, we consider the complex-valued measurable functions f, γ, φ, ..., each determined only a.e., and each belonging to all L_p-classes simultaneously, $1 < r < \infty$.

We donote (i) by $F = \{f\}$ a ring of functions with complex constants and ordinary multiplication and closed under the involution: if $f \equiv f_1 + if_2 \in F$ then also $\bar{f} \equiv f_1 - if_2 \in F$ and hence also $f_1, f_2 \in F$; (ii) by $\Gamma = \{\gamma\}$ a subring of F also closed under this involution; (iii) by $\Phi = \{\varphi\}$ another subring but this one *not* closed under the involution; (iv) by $\bar{\Phi} = \{\bar{\varphi}\}$ the ring of elements conjugate complex to those of Φ, and we make the following interlocking assumptions; (v) if $\gamma \in \Gamma$, $\varphi \in \Phi$ then $\gamma\varphi \in \Phi$, and thus, by involution in Γ, also $\gamma\bar{\varphi} \in \bar{\Phi}$, (vi) for all $\varphi \in \Phi$,

$$\int \varphi d\sigma = 0.$$

Lemma 1. *If $\gamma \in \Gamma$, $\varphi, \psi \in \Phi$ and*

$$\gamma + \varphi + \bar{\psi} = 0$$

then $\gamma = \varphi = \bar{\psi} = 0$.

In fact, $(\gamma + \varphi)\psi + \psi\bar{\psi} = 0$. Now $(\gamma + \varphi)\psi \in \Phi$ hence by (vi) its integral is 0 and therefore $\int \psi\bar{\psi} d\sigma = 0$ and $\psi = 0$. Similarly $\varphi = 0$, and thus $\gamma = 0$.

We now add the general assumptions: (vii) each $f \in F$ can be represented (uniquely by Lemma 1) as

$$f = \gamma + \varphi + \bar{\psi}, \tag{1}$$

and (viii) the vector space F is Banach-dense in each L_p, $1 < p < \infty$.

Representation (1) gives rise to linear operators

$$\gamma = Af, \qquad \varphi = Bf, \qquad \psi = B\bar{f}$$

which enter into the following proposition.

Theorem 1 (M. Riesz). *If for each $p > 1$ there is a finite constant M_p such that*

$$\|Af\|_p \leq M_p \cdot \|f\|_p, \qquad 1 < p < \infty$$

then there is another constant N_p, which depends only on M_p and p, such that

$$\|Bf\|_p \leq N_p \cdot \|f\|_p, \qquad 1 < p < \infty.$$

This version is an axiomatic generalization of a previous generalization of ours[1] of a basic theorem of M. Riesz for functions in one variable. As before[1] we again introduce the real valued elements in F,

$$f = \gamma + \varphi + \bar{\varphi} \qquad \gamma = \bar{\gamma}$$

and for these the operator

$$\tilde{f} = Cf \equiv i(\bar{\varphi} - \varphi),$$

and it again[1] suffices to prove that there are finite bounds R_p for which

$$\|\tilde{f}\|_p \leq R_p \cdot \|f\|_p.$$

Since $f + i\tilde{f} = \gamma + 2\varphi$ we have $\int (f + i\tilde{f})^{2k} d\sigma = \int \gamma^{2k} d\sigma$, and hence for $k = 1, 2, 3, \ldots$,

$$\int \tilde{f}^{2k} d\sigma - \binom{2k}{2} \int \tilde{f}^{2k-2} f^2 d\sigma + \binom{2k}{4} \int \tilde{f}^{2k-4} f^4 d\sigma - \ldots = (-1)^k \int \gamma^{2k} d\sigma.$$

By Holder inequality the quotient

$$Y = \|\tilde{f}\|_{2k} : \|f\|_{2k}$$

does not exceed the largest root of

$$Y^{2k} - \binom{2k}{2} Y^{2k-2} - \binom{2k}{4} Y^{2k-4} - \ldots - \left(1 + M \binom{2k}{2k}\right) = 0$$

and this establishes the existence of R_p for $p = 2, 4, 6, 8, \ldots$. By assumption (viii) the existence of R_p now follows for $2k < p < 2k + 2$ from double convexity theory,[2] and finally for $1 < p < 2$ by duality from

$$\int f . Cg \, d\sigma = - \int Cf . g \, d\sigma.$$

Turning to another but cognate subject, we now assume that our underlying point space is a compact Hausdorff space, that $d\sigma$ indicates a Borel measure of total measure 1 and that for any open set the measure is nonzero. About the ring $F = \{f\}$ we assume more precisely than above that all f are continuous, and of $\Gamma = \{\gamma\}$ we assume more precisely that it shall consist of all constant functions, and we again introduce Φ as before.

THEOREM 2 (Szegö-Kolmogoroff-Krein). *If μ is a non-negative measure on the Borel sets with*

$$d\mu = w(x) \, d\sigma + d\mu_\sigma$$

where $w(x)$ is summable and $d\mu_\sigma$ is singular then

$$\exp \left\{ \int \log w \, d\sigma \right\} = \inf_{\varphi \in \Phi} \int |1 + \varphi|^2 \, d\mu$$

and the left side is to be interpreted as 0 if

$$\int \log w \, d\sigma = - \infty.$$

This version of the theorem is nothing but a straightforward axiomatization of a recent important multi-variable version due to Helson and Lowdenslager[3] and the proof is identical. Other theorems of theirs also continue valid, as the following ones.

THEOREM 3. *If f is "analytic," that is*

$$f = b + \varphi$$

then

$$\int \log |f| \, d\sigma \geq \log |b|.$$

This also holds if f is an L_1-limit of our analytic functions and $b = \int f d\sigma$.

THEOREM 4. *Let $w(x)$ be non-negative and summable. It has a representation*

$$w = |b + \chi|^2, \qquad b \neq 0$$

where χ is in the L_2-closure of Φ if and only if

$$\int \log w \, d\sigma > -\infty.$$

THEOREM 5. *Let $f \in L_1$ and*

$$f = a + \chi, \qquad a \neq 0,$$

where χ is in the L_1-closure of Φ. There exist functions $g = b + \chi_1$, $h = c + \chi_2$ with χ_1, χ_2 in the L_2-closure of Φ such that $f = g \cdot h$.

THEOREM 6 (Beurling). *Let H be the family*

$$a + \chi$$

with χ in the L_2 closure of Φ. For $f \in H$ let C_f be the smallest closed linear manifold containing

$$(b + \varphi)f$$

for all constants b and all $\varphi \in \Phi$, then $C_f = H$ if and only if

$$\int \log |f| d\sigma = \log \left| \int f d\sigma \right| > -\infty.$$

* This research was supported by the United States Air Force through the Office of Scientific Research of the Air Force Research and Development Command.

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A COVERING HOMOTOPY THEOREM AND THE CLASSIFICATION OF G-SPACES

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Communicated by Deane Montgomery, April 3, 1959

In this note we shall describe a classification theory for compact Lie transformation groups with a finite number of orbit types. The classification is analogous to the classification of principal bundles in terms of universal bundles and their classifying spaces, the latter in fact being the special case in which there is only one orbit type which is equivalent to the group acting on itself by translation. It is expected that details and proofs will appear in a Memoir of the American Mathematical Society.

Let G be a compact Lie group with identity e. We write $H \subset G$ to mean that H is a closed subgroup of G, and we write (H) for the set $\{gHg^{-1} | g \in G\}$ of subgroups

of G conjugate to H. Such a set we call a *G-orbit type*. A *G-space* is a locally compact, second countable, Hausdorff space with a fixed action of G on X. We write Gx for the orbit of $x \in X$ under G and G_x for the isotropy group at x. The set of all isotropy groups on a single orbit is an orbit type which we call the type of the orbit. We write $X_{(H)}$ for the union of all orbits in X of type (H) and $\tilde{X}_{(H)}$ for the set orbits of X of type (H). If X/G is the orbit space of X (a locally compact, second countable Hausdorff space) and Π_X the natural map of $X \to X/G$ then $\tilde{X}_{(H)} = \Pi_X(X_{(H)})$. A map f of a G-space X into a G-space Y is called *isovariant* if it is equivariant (i. e., $f(gx) \equiv gf(x)$) and one-to-one on each orbit. For such a map we have $G_{f(x)} \equiv G_x$ so that $f(X_{(H)}) \subset Y_{(H)}$ and $\tilde{f}(\tilde{X}_{(H)}) \subseteq \tilde{Y}_{(H)}$ where $\tilde{f}: X/G \to Y/G$ is the induced map which satisfies $\tilde{f}\Pi_X = \Pi_Y f$. We make the product $X \times I$ of a G-space X with the unit interval into a G-space by $g(x, t) = (gx, t)$ and a homotopy $f: X \times I \to Y$ of an isovariant map $f_0: X \to Y$ is called an *isovariant* homotopy of f_0 if it is isovariant in this G-space structure. With a natural identification $(X \times I)/G = X/G \times I$, and the induced map $\tilde{f}$ of an isovariant homotopy of f_0 (which is clearly a homotopy of $\tilde{f}_0$) clearly satisfies $\tilde{f}(\tilde{X}_{(H)} \times I) \subseteq \tilde{Y}_{(H)}$. The covering homotopy theorem says that this condition is also sufficient for a homotopy $\tilde{f}$ of $\tilde{f}_0$ to be the induced map of an isovariant homotopy of f_0.

COVERING HOMOTOPY THEOREM. *Let X and Y be G-spaces and $f_0: X \to Y$ an isovariant map. If $\tilde{f}: X/G \times I \to Y/G$ is a homotopy of the induced map $\tilde{f}_0$ of f_0 which satisfies $\tilde{f}(\tilde{X}_{(H)} \times I) \subseteq \tilde{Y}_{(H)}$ for all G-orbit types (H) then $\tilde{f}$ is the induced map of an isovariant homotopy of f_0.*

Now let Σ be a finite collection of G-orbit types. We assume henceforth that all G-spaces X satisfy $X = \bigcup\{X_{(H)} \mid (H) \in \Sigma\}$, and hence are Σ-spaces, as are their orbit spaces, in the following sense. A *Σ-space* is a locally compact, second countable space Z with a fixed partition $\{Z_{(H)}\}$ into subsets indexed by Σ. We make $Z \times I$ into a Σ-space by $(Z \times I)_{(H)} = Z_{(H)} \times I$. A map f of a Σ-space Z into a Σ-space X is called a *Σ-map* if $f(Z_{(H)}) \subset X_{(H)}$ for all $(H) \in \Sigma$, and a Σ-equivalence if, in addition, it is a homeomorphism of Z onto X. If f_0 and f_1 are two Σ-maps of Z into X they are called *strongly Σ-homotopic* if there is a Σ-map $f: Z \times I \to X$ which is a homotopy of f_0 with f_1. If f_0 is strongly Σ-homotopic to $f_1 \circ h$ where h is some Σ-equivalence of Z with itself then f_0 and f_1 are called *weakly Σ-homotopic*. A *G-space over the Σ-space Z* is a triple (Y, Z, h) where Y is a G-space and h is a Σ-equivalence of Y/G with Z. It is called *weakly equivalent* to a second G-space over Z (Y', Z, h') if there is an equivariant homeomorphism f of Y onto Y', and *strongly equivalent* (to Y', Z, h') if, in addition, f can be so chosen that $h' \circ f \circ h^{-1}$ is the identity map of Z.

If Z is a Σ-space, X a G-space and $f^*: Z \to X/G$ a Σ-map we define a G-space (Y, Z, h) over Z denoted by $f^{*-1}(X)$ and called the *G-space over Z induced by f^** as follows:

$$Y = \{(x, z) \in X \times Z \mid \pi_X(x) = f^*(z)\}$$
$$g(x, z) = (gx, z)$$
$$h \circ \pi_X(x, z) = Z.$$

It follows from the Covering Homotopy Theorem that

THEOREM. *$f^{*-1}(X)$ depends to within weak (strong) equivalence only on the weak (strong) Σ-homotopy class of f^*.*

If n is a positive integer then a G-space X is called (Σ, n)-*universal* if given any isovariant map f of a closed invariant subspace of a G-space Y into X there exists an extension of f to an isovariant map of Y into X, provided $\dim (Y/G) \leq n$.

CLASSIFICATION THEOREM. *Let X be a $(\Sigma, n+1)$-universal G-space and let Z be a Σ-space of dimension $\leq n$. Then the map which takes the strong (weak) Σ-homotopy class of $f^* \to$ strong (weak) equivalence class of $f^{*-1}(X)$ is a one-to-one correspondence between the strong (weak) Σ-homotopy classes of Σ-maps of Z into X/G and the strong (weak) equivalence classes of G-spaces over Z.*

To give content to the above theorem we must be able to construct (Σ, n)-universal G-spaces. This is done as follows. Given G-spaces $X_1, \ldots, X_n$ let $X_1 \circ \ldots \circ X_n$ denote their join (see J. Milnor, The construction of universal bundles II, *Annals of Math.*, **63**, no. 3, May, 1956) made into a G-space by $g(t_1x_1, \ldots, t_nx_n) = (t_1gx_1, \ldots, t_ngx_n)$. We define the *reduced join* $X_1^* \ldots {}^* X_n$ of $X_1, \ldots X_n$ to be the invariant subset of their join consisting of those points $(t_1x_1, \ldots, t_nx_n)$ for which the set of isotropy groups G_{x_i} for which $t_i \neq 0$ has a smallest element under inclusion. We denote the k-fold join (reduced join) of a space X by $X^{(ok)}(X^{(*k)})$. Let $\Sigma = ((H_1), \ldots, (H_m))$ and let n be any positive integer. It follows from Lemma 2.3 (Milnor, *loc. cit.*) that we can find integers $k_1, \ldots, k_m$ such that $(N(H_i)/(H_i)^{(ok_i)}$ is n-connected, where $N(H_i)$ is the normalizer of H_i in G. Then

THEOREM. $(G/H_1)^{(*k_1)} * \ldots * (G/H_m)^{(*k_m)}$ *is* (Σ, n)-*universal.*

ORTHOGONAL LATIN SQUARES

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Communicated by A. A. Albert, April 20, 1959

1. Introduction.—The principal purpose of this note is to display the first pair of orthogonal latin squares of order 10. A *latin square* of order n is an $n \times n$ matrix with exactly n distinct symbols, each symbol in each row (necessarily only once) and each symbol in each column (once). Two latin squares of order n are called *orthogonal* if each ordered pair of symbols occurs (once) in some cell, the first symbol in the first latin square and the second symbol in the second latin square. More generally, several latin squares of order n are a *mutually* (or *pairwise*) *orthogonal* set if each pair of latin squares is orthogonal. Unlike the somewhat similar magic squares, orthogonal latin squares are today no isolated combinatorial curiosity. For a set of $n-1$ mutually orthogonal latin squares of order n is equivalent to an affine plane of order n. In turn, an affine plane determines a projective plane of the same order; and a projective plane is the completion of at least one affine plane of like order. The nonspecialist is referred to expository papers[1-3] and their bibliographies.

Eulre[4] introduced the concept of pair of orthogonal latin squares (also called Greco-Latin squares). He obtained several results, and conjectured that no pair exists of any order $n \equiv 2 \pmod 4$. Tarry[5] demonstrated by a lengthy case-by-case argument the truth of Euler's conjecture for order 6; his result made the conjecture

seem more plausible. Bose and Shrikhande[6] have very recently obtained the first counterexamples to Euler's conjecture; they constructed pairs of orthogonal latin squares of infinitely many orders $n \equiv 2 \pmod 4$, their lowest order being 22.

Order 10 is of considerable interest in itself, being the lowest order for which the existence of a projective plane remains undecided, and having been also the lowest undecided order for existence of a pair of orthogonal latin squares. Considerable effort has been expended in searching by digital computer for a pair of orthogonal latin squares of order 10, but machines have proved too slow to cope with a search of such magnitude.

Section 2 contains a theorem and a corollary yielding construction of pairs of orthogonal latin squares of infinitely many orders $n \equiv 2 \pmod 4$, the first order being 10. In Section 3 a pair of orthogonal latin squares of order 10 is displayed. In Section 4 are some concluding remarks.

2. The Theorem.—First will be proved a lemma, which is familiar to some, but is apparently not in the literature. (This lemma in more general form is in reference 7).

LEMMA. *A pair of orthogonal latin squares of order n is equivalent to a set of n^2 ordered quadruples $(a_{i1}, a_{i2}, a_{i3}, a_{i4})$, $i = 1, \ldots, n^2$, with elements a_{ij} the numbers $1, \ldots, n$, and such that for each pair u, v of integers, $1 \leq u < v \leq 4$, and each pair x, y of numbers from $1, \ldots, n$, $a_{iu} = x$ and $a_{iv} = y$ both hold for some i $(i = 1, \ldots, n^2)$.*

Proof: There being exactly n^2 ordered quadruples in the set, $a_{iu} = x$ and $a_{iv} = y$ are satisfied for a unique i. Associate the n^2 ordered quadruples with cells of two $n \times n$ matrices, a_{i1} and a_{i2} chosen as the row and column indices, respectively, a_{i3} and a_{i4} as the digits in the designated cell of the first and second matrix, respectively. The conditions imply that the matrices are orthogonal latin squares. For when u and v are 1 and 2, each cell is accounted for. When $u = 1$ and v is 3 or 4, each row of the appropriate matrix contains each digit—only once, of course. Similarly when $u = 2$ and v is 3 or 4, the same holds on columns. When u and v are 3 and 4, each ordered pair of digits occurs (exactly once) in some cell of the matrices. The converse construction of the set of ordered quadruples from the pair of orthogonal latin squares is carried out similarly.

THEOREM. *There exists a pair of orthogonal latin squares of order $(3q - 1)/2$, where q is a prime-power, and $q \equiv 3 \pmod 4$. (While there exists a pair of orthogonal latin squares of order 4, the construction below fails for $q = 3$.)*

Proof: A set of ordered quadruples satisfying the conditions of the Lemma will be constructed. In all four positions, the $(3q - 1)/2$ symbols are chosen as the q elements of $GF[q]$, and X_i, $i = 1, \ldots, (q - 1)/2$. Form the ordered quadruples,

$$(X_i, a, r^{2i} + a, r^{2i}(r + 1) + a)$$
$$(r^{2i}(r + 1) + a, X_i, a, r^{2i} + a)$$
$$(r^{2i} + a, r^{2i}(r + 1) + a, X_i, a)$$
$$(a, r^{2i} + a, r^{2i}(r + 1) + a, X_i),$$

where $i = 1, \ldots, (q - 1)/2$, a ranges over the elements of $GF[q]$, and r is a fixed primitive element (i.e., a generator of the cyclic multiplicative group) of $GF[q]$. The above ordered quadruples are related by cyclic permutation of the four positions. Selecting all pairs i, a generates a list of $4 \cdot q \cdot (q - 1)/2 = 2q(q - 1)$ ordered quadruples. It is easily checked that each X_i occurs once in the list with each element of $GF[q]$ in each ordered pair of positions. No two like or distinct X_i occur in

any of these quadruples; no element of $GF[q]$ is repeated in a quadruple. Since $(r^{2i} + a) - a = r^{2i}$ ranges over (nonzero) square elements of $GF[q]$, and $\{r^{2i}(r + 1) + a\} - (r^{2i} + a) = r^{2i+1}$ ranges over nonsquare elements of $GF[q]$, each pair of distinct elements of $GF[q]$ occurs once in each adjacent pair of positions once in either order—here the first and fourth positions are considered adjacent. Since $q \equiv 3 \pmod 4$, -1 is a nonsquare in $GF[q]$. It follows that $\{r^{2i}(r + 1) + a\} - a$ and $a - \{r^{2i}(r + 1) + a\}$ are one square and one nonsquare in $GF[q]$. Hence each ordered pair of distinct elements of $GF[q]$ occurs once in the first and third positions; once in the second and fourth positions. (Since -1 is the only primitive element of $GF[3]$, and $-1 + 1 = 0$, the construction fails for $q = 3$.)

Form the ordered quadruples, (a, a, a, a), where again a ranges over the elements of $GF[q]$. These q quadruples account for one occurrence in each pair of positions of each a with itself. Finally, generate a set of $(q - 1)^2/4$ ordered quadruples equivalent to any pair of orthogonal latin squares of order $(q - 1)/2$, with the X_i in all four positions. (A pair of orthogonal latin squares of any odd order exists.) In this latter set of ordered quadruples, each pair of like or distinct X_i occurs in each pair of positions once. Collectively, the three classes of ordered quadruples form a set of $\{(3q - 1)/2\}^2$ satisfying the conditions of the Lemma.

By the celebrated theorem of Dirichlet, there exist infinitely many prime-powers $q \equiv 7 \pmod 8$. Each such q in the above construction produces a pair of orthogonal latin squares of order $(3q - 1)/2 \equiv 10 \pmod{12}$.

COROLLARY. *There exists a pair of orthogonal latin squares of order any odd multiple of any order constructed in the Theorem.*

Proof: MacNeish[8] has shown the following by a straightforward constructive method: If there exist sets of t mutually orthogonal latin squares of orders m and n, respectively, then there exists a set of t mutually orthogonal latin squares of order mn. Specializing to $t = 2$, and noting a pair of latin squares exists of any odd order, the Corollary is immediate.

3.—A pair of orthogonal latin squares of order 10, constructed from the Theorem is displayed. Here $q = 7$, $r = 3$, and digits 7, 8, 9 correspond to the X_i. The horizontal and vertical divisions are intended to bring out relations.

0 4 1 7 2 9 8	3 6 5	0 7 8 6 9 3 5	4 1 2
8 1 5 2 7 3 9	4 0 6	6 1 7 8 0 9 4	5 2 3
9 8 2 6 3 7 4	5 1 0	5 0 2 7 8 1 9	6 3 4
5 9 8 3 0 4 7	6 2 1	9 6 1 3 7 8 2	0 4 5
7 6 9 8 4 1 5	0 3 2	3 9 0 2 4 7 8	1 5 6
6 7 0 9 8 5 2	1 4 3	8 4 9 1 3 5 7	2 6 0
3 0 7 1 9 8 6	2 5 4	7 8 5 9 2 4 6	3 0 1

1 2 3 4 5 6 0	7 8 9	4 5 6 0 1 2 3	7 8 9
2 3 4 5 6 0 1	8 9 7	1 2 3 4 5 6 0	9 7 8
4 5 6 0 1 2 3	9 7 8	2 3 4 5 6 0 1	8 9 7

4. Remarks.—The author is indebted to Professors Bose and Shrikhande for the crucial idea[6] of applying a group of automorphisms to the set of ordered quadruples *except* for a subset equivalent to a pair of orthogonal latin subsquares. The author failed to handle Euler's conjecture with a group of automorphisms over all

ordered quadruples; he did, however, disprove[7] MacNeish's[8] generalization of the Euler conjecture.

The above pair of orthogonal latin squares of order 10 cannot be extended to a set of 9 mutually orthogonal latin squares of order 10, generating a plane of order 10. For the plane would have a subplane of order 3 formed by the set of $3 - 1 = 2$ orthogonal latin squares of order 3; this is impossible by a theorem of Bruck.[9] Whether the techniques referred to[7,6] and this note will lead to more significant results can hardly be predicted so soon. There is less reason than before to believe that all finite projective planes are of prime-power orders. The author hopes that more effort will now be put into attempts to construct projective planes of nonprime-power orders, and that someone will succeed.

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*A DEMONSTRATION OF ANTIBODY ACTIVITY ON MICROSOMES**

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Communicated by Colin M. MacLeod, April 13, 1959

Lymph nodes are well known to be the principal site for the synthesis of antibodies.[1] Since there is a growing body of evidence that an important stage in protein synthesis involves the condensation of amino acids on microsomes,[2,3] it was of interest to determine whether antibody activity could be demonstrated on microsomes isolated from the lymph nodes of antigenically stimulated animals. The assay devised for this purpose was based on the reversible binding of a soluble antigen by antibodies which were insoluble due to their association with microsomal particles. I^{131} labeled antigen was used to provide an indicator of high sensitivity. In order to increase the opportunities for evaluating the specificity of the assay procedure, the immune system used was specific for a simple chemical group (2,4-

dinitrophenyl). DNP-BγG[4] was used for induction of antibody formation, I^{131}-DNP-BSA was employed as the soluble test antigen, and ϵ-DNP-lysine served as the soluble hapten for displacing reversibly bound antigen.

Methods and Materials.—Materials: DNP-BγG and DNP-BSA were prepared with DNP groups on ϵ-NH_2 groups of lysine residues as described previously.[5,6] These proteins contained 65 and 7 moles DNP per mole protein, respectively. ϵ-DNP-lysine was prepared by the procedure of Porter.[7] DNP-BSA and BSA were iodinated[8] with I^{131}, using KI as carrier to improve the yield. The iodinated proteins had about 10 moles iodine per mole BSA.

Immunization of animals: Guinea pigs (about 350 gm) were injected in each of their foot-pads with 0.1 ml. of DNP-BγG, or BγG, in Freund's adjuvant.[9] Each animal received a total of 1.2 mg protein.

Preparation of microsomes: Three to six weeks after immunization, the animals were sacrificed by a blow on the head and their axillary, inguinal, and popliteal lymph nodes were removed. The following procedures were performed at 3°. Lymph nodes, trimmed of excess fat, were minced and suspended in the medium described by Littlefield and Keller,[10] using 2.3 ml/mg wet weight tissue. Following homogenization (ground glass homogenizer) the suspension was centrifuged at $15{,}000 \times g$ for 10 min. After discarding surface lipid the supernatant was removed by aspiration and centrifuged again for 45 min at $15{,}000 \times g$. The surface lipid was discarded and the supernatant removed as above. The supernatant was divided among several tubes so that each contained a volume of supernatant which was derived from about 2 gm wet weight of lymph nodes. The tubes were centrifuged at $105{,}000 \times g$ for 60 min and the supernatants were discarded by decantation. The pellets were rinsed 3 times (by overlay and decantation) with ice-cold homogenizing solution. The pellets finally obtained are referred to as microsomes.[10] The above procedure was also used to prepare guinea pig liver microsomes.

Experimental procedure: Figure 1 outlines the basic procedure. The medium used to resuspend the microsomes (BSA-medium) was the homogenizing medium enriched with 5 mg/ml BSA. The difference between I^{131} in the control tube and its paired experimental tube (Fig. 1) represents the I^{131}-antigen specifically displaced by ϵ-DNP-lysine. Each entry in Tables 1 and 2 represents the difference between a pair of such tubes. In a typical experiment the control eluate had 2,740 cpm/1.7 mg microsome protein, and the experimental eluate had 23,300 cpm/1.7 mg microsome protein. From the specific activity of the I^{131}-antigen (210 cpm/mμg), this difference corresponds to 58 mμg I^{131}-DNP-BSA/mg microsome protein (Table 1, 1st line). The amount of I^{131}-DNP-BSA remaining on the microsomes after elution by $1 \times 10^{-3}M$ ϵ-DNP-lysine was about 8 mμg/mg microsome protein.

I^{131} was determined with a scintillation counter. Protein in the final microsomal pellet (Step 5) was isolated following extraction of lipids and nucleic acids.[11] The isolated protein was dissolved in $5.2M$ acetic acid, and its absorbance at 280 mμ was determined. In this solvent, microsomal protein had a ratio of absorbances at 280/260 mμ of 1.6, and at 280 mμ $E^{1\%}_{1cm}$ was 13.6.

Results.—The following results, which are summarized in Table 1, demonstrate the presence of anti-DNP antibody activity on microsomes:

(*a*) ϵ-DNP-lysine dissociates I^{131}-DNP-BSA from the lymph node microsomes of animals immunized with DNP-BγG, but not from the lymph node microsomes of

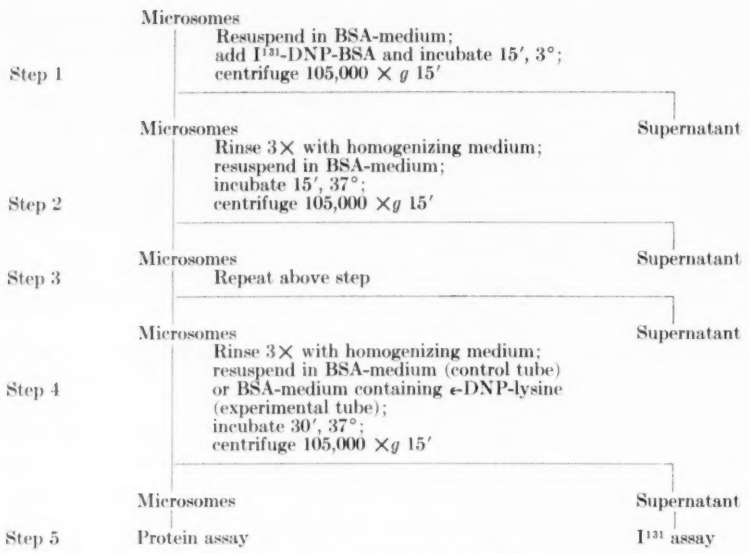

FIG. 1.—Experimental procedure.

guinea pigs immunized with BγG (experiments 1 and 3). ϵ-DNP-lysine has a high affinity for anti-DNP antibodies and specifically dissolves precipitates formed by such antibodies and homologous antigen.[12,13]

(*b*) Incubation of microsomes with DNP-BγG or with ϵ-DNP-lysine prior to incubation with I^{131}-DNP-BSA inhibited the specific binding of the I^{131}-DNP-BSA, as shown by the subsequent failure of ϵ-DNP-lysine to specifically elute radioisotope (experiment 1). However, incubation of microsomes with BγG (not containing DNP groups) did not interfere with the specific binding of I^{131}-DNP-BSA (experiment 1).

(*c*) ϵ-DNP-lysine, at low concentrations, specifically inhibits the interaction of anti-DNP antibodies with a DNP-protein antigen (50 per cent inhibition of precipitation at $5 \times 10^{-6} M$).[12] Accordingly, the sensitivity of the microsome: I^{131}-DNP-BSA complex to this hapten was determined. As shown in experiment 2, ϵ-DNP-lysine is nearly as effective at $5 \times 10^{-6} M$ as at $1 \times 10^{-3} M$ in displacing I^{131}-DNP-BSA.

(*d*) 2,4-dinitrophenol is much less effective than ϵ-DNP-lysine as regards the specific dissociation of immune precipitates (anti-DNP antibodies with DNP-protein),[12] and is also less effective in respect to the dissociation of I^{131}-DNP-BSA from microsomal antibodies (experiment 2).

(*e*) As final evidence for the immunologic specificity of the system employed, it should be noted that when I^{131}-BSA was used as the indicator antigen, in place of I^{131}-DNP-BSA, subsequent treatment with ϵ-DNP-lysine failed to elute radioactivity (experiment 2).

Blood containing anti-DNP antibodies is present in the lymph nodes removed from guinea pigs immunized with DNP-BγG. The possibility that the antibody

TABLE 1

SPECIFICITY OF MICROSOMAL ANTIBODIES

Exp. No.	Immunized with	Microsomes: Additions*	Microsomes: Incubated with†	Microsomes: Eluted with	I^{131}-Protein Eluted,§ mμg/mg Microsome Protein
1	DNP-BγG	....	I^{131}-DNP-BSA	ε-DNP-lysine, $1 \times 10^{-3}M$	58
		ε-DNP lysine	"	" "	0
		DNP-BγG	"	" "	0
		BγG	"	" "	53
2	DNP-BγG	....	I^{131}-DNP-BSA	ε-DNP-lysine, $1 \times 10^{-3}M$	45
		....	"	" , $5 \times 10^{-6}M$	40
		....	"	" , $5 \times 10^{-6}M$	34
		....	"	Dinitrophenol, $2 \times 10^{-3}M$‡	27
		....	I^{131}-BSA	ε-DNP-lysine, $1 \times 10^{-3}M$	0
3	BγG	....	I^{131}-DNP-BSA	ε-DNP-lysine, $1 \times 10^{-3}M$	0

The experimental procedure is described in Materials and Methods.

* Fifteen minutes before addition of I^{131}-DNP-BSA the indicated substances were added to the microsomes in a total volume of 5 ml: ε-DNP-lysine (5 μmoles), DNP-BγG (20 μg protein, 7.2 mμmoles DNP), BγG (20 μg protein).

† I^{131}-DNP-BSA added as 8 μg protein (0.8 mμmoie DNP); maximally, 1 mμg was 500 cpm. I^{131}-BSA was added as 8 μg protein; maximally, 1 mμg was 350 cpm.

‡ Estimated to be $1 \times 10^{-3}M$, due to binding by bovine serum albumin in the medium.[16]

§ 0 corresponds to <1 mμg I^{131} protein eluted/mg microsomal protein.

TABLE 2

EXTENT OF CONTAMINATION OF MICROSOMES BY SERUM ANTIBODIES

Exp. No.	Immunized with	Microsomes: Source	Serum Added*, %	I^{131}-DNP-BSA Eluted,†‡ mμg/mg Microsome Protein
4	DNP-BγG	Lymph node	..	91
		Liver	..	3
5	BγG	Lymph node	0	0
		"	10	3§
		"	30	13
		"	60	23

The experimental procedure is described in Materials and Methods.

* The serum was obtained from the animals used in experiment 4 and contained 164 μg anti-DNP antibody N/ml. The amount of serum added directly to microsomes was based on the wet weight of the lymph nodes (i.e., 10 per cent serum means that 0.1 ml. serum was added to the microsomes obtained from 1.0 gm lymph node).

† I^{131}-DNP-BSA added as per Table 1. Eluted with $1 \times 10^{-3}M$ ε-DNP-lysine.

‡ 0 corresponds to <1 mμg I^{131}-DNP-BSA eluted/mg microsomal protein.

§ Essentially the same results were achieved when similar lymph nodes were homogenized in the presence of an equivalent amount of anti-DNP antibody.

activity associated with microsomes is due to contamination of these particles by antibodies adsorbed from serum is excluded by the results given in Table 2.

Since liver would be expected to contain at least as much blood as lymph nodes, microsomes were prepared from the livers and lymph nodes of the same DNP-BγG immunized animals. Antibody activity of the liver microsomes was only 4 per cent of the activity of lymph node microsomes (experiment 4). Because this experiment is subject to the limitation that microsomes from these tissues are not necessarily equal as regards the adsorption of antibody, pooled serum collected from the animals of experiment 4 was incubated with lymph node microsomes derived from animals immunized with BγG. It may be seen (experiment 5) that the adsorption of antibodies by microsomes is linear with antibody concentration, but slight in extent. In order for serum contamination to account for the I^{131}-DNP-BSA binding by lymph node microsomes of DNP-BγG immunized animals (Table 2), an impossible degree of contamination would be required (1 gm wet weight lymph node contaminated by about 4.8 ml whole blood).

A further indication that the antibody activity of microsomes is not due to con-

tamination by soluble antibody from serum or lymphatic tissue was provided by the following experiment: 6.3 gm lymph node of BγG immunized guinea pigs (*A*) and 7.2 gm lymph node of DNP-BγG immunized guinea pigs (*B*) were homogenized, and the supernatants remaining after sedimentation of microsomes were exchanged (i.e., supernatant *A* added to microsomes *B* and vice versa), and the microsomes were suspended in the foreign supernatant. The microsomes were then collected by centrifugation and processed as outlined in Fig. 1. The specific elution by ϵ-DNP-lysine yielded 590 cpm from the microsomes of BγG immunized animals and 7,830 cpm from the microsomes of DNP-BγG immunized animals.

Discussion.—The experiments described demonstrate antibody activity on particles with the sedimentation characteristics of microsomes. This activity was induced in lymph nodes which drain sites of injection of DNP-BγG, and was specific for the DNP group. The possibility that the antibody activity associated with microsomes was an artifact arising from adsorption of soluble antibody was excluded by a variety of experiments.

By treatment of chicken liver microsomes with desoxycholate, Peters[14] isolated a protein which was indistinguishable from chicken serum albumin as regards immunologic and some physical and chemical characteristics. Whether the antibody activity induced on lymph node microsomes is due to a protein which is identical with serum antibodies remains to be established. For this and other reasons, it undoubtedly will be of considerable interest to define the nature of the bonds which link antibodies to microsomes. Although there is uncertainty as to whether microsomal and serum antibodies are structurally identical, it is clear that their specific binding sites are qualitatively similar. Thus, ϵ-DNP-lysine and 2,4-dinitrophenol have about the same relative capacities in respect to the displacement of I^{131}-DNP-BSA from microsomal antibody and the dissolution of specific precipitates made with serum anti-DNP antibodies.[12]

The demonstration of antibody activity on microsomes cannot be construed to mean that antibodies acquire their definitive fine structure on microsomes. The observations described would be equally consistent with the synthesis of antibodies at some other site with translocation to microsomes.

Intracellular antibodies have been elegantly demonstrated by the fluorescent staining procedure of Coons *et al.*,[15] but the resolving power of this method does not permit a distinction to be made between soluble and microsomal antibody. While it is tempting to speculate regarding the possible biological significance of microsomal antibody it seems unwarranted at this time to do more than record our observations.

Summary.—When 2,4-dinitrophenyl-bovine γ globulin is injected into guinea pigs there is induced on the microsomes of regional lymph nodes an antibody activity which is specific for the 2,4-dinitrophenyl group. This antibody activity cannot be attributed to contamination of microsomes by soluble antibodies contained in lymph nodes.

We are grateful to John Courtney and Richard Pinkston for valuable technical assistance.

* This work was supported, in part, by grants from the National Institutes of Health (E-1363, E-2130, C-3958), and by a contract with the Research and Development Division, Office of the

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*ISOLATION AND STUDY OF MUTANTS FROM MAMMALIAN CELLS IN CULTURE**

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Communicated by Curt Stern, April 3, 1959

The observation that a single mammalian cell can be grown to yield a colony of cells[1] has made it possible to search for mutants[2] in a population of cultured cells. Mutants resistant to the antibiotic puromycin (6-dimethylamino-9-[3′-deoxy-3′-(*p*-methoxy-L-phenylalanyl-amino)-β-D-ribofuranosyl]-purine [3, 4]) or the purine analogue 8-azaguanine have now been obtained with a fibroblast-like cell, AMK 2-2.[5] Interestingly, as with bacteria,[6] increased resistance to toxic agents may or may not occur in relatively small steps.

The purpose of this report is to describe the isolation of mutant mammalian cells, to indicate that they can be manipulated in procedures comparable to some of those employed in the study of microbial genetics, and to detail some of the properties of the mutants.

Materials.—The basal medium contained the amino acids, inorganic salts, glucose, and antibiotics suggested by Healy, Fisher, and Parker,[7] and the vitamin mixture of Eagle.[8] The *growth medium* contained in addition 15 per cent beef serum. For cloning, a mixture of human (15 per cent) and horse serum (5 per cent) (*cloning medium*) was substituted for the bovine serum. Since unheated human serum causes rapid lysis of AMK cells, human sera were heated (55°, 30 min) before use.

Puromycin was a gift from Dr. J. P. English, Lederle Laboratories. 8-azaguanine was obtained from the California Foundation for Biochemical Research and from the Nutritional Biochemical Corporation. The samples were spectrophotometrically indistinguishable at various pH levels.

The test tubes used for assay were 16 × 125 mm in size and were fitted with rubber-lined, plastic screwtops; Petri dishes were 60 mm in diameter.

Culture Methods.—Unless otherwise indicated, cells were grown in suspension in 50-ml test tubes fitted with silicone stoppers. To prevent the cells from settling out and adhering to the glass, cultures were incubated horizontally in a drum revolving at 40 rpm.[9]

To prepare a suspended culture from a colony, the colony was encircled with a stainless steel ring and the cells were detached from the Petri dish by aspirating several times with a capillary pipette. They were then transferred to a screwtop test tube containing 2 ml of cloning medium. When confluent, the cells were detached with a rubber spatula and allowed to grow (5 ml of growth medium) on the surface of a 50-ml test tube incubated in an almost horizontal position. Finally, fresh growth medium (10–20 ml) was added, the cells were detached into the medium, and the tube was placed in a revolving drum.

TABLE 1*

EFFECT OF PUROMYCIN ON VIABLE PARENT AND MUTANT CELLS

Puromycin, μg per ml	Culture			
	Pms^{-1}	*Pms*$^{-2}$	*Pmr*$^{-1}$	*Pmr*$^{-2}$
	Cell Number in Thousands			
0	376	384	475	417
2	0	211	...	...
4	...	0	...	...
8	...	...	481	...
12	...	...	435	...
16	...	...	106	336
24	...	...	0	356
40	...	...	...	333
80	...	...	...	31
120	...	...	...	3

* The cells were obtained from exponentially-growing, suspended cultures of clonal descendants. One milliliter aliquots of suspensions in growth medium (10^8 cells per ml) were added to test tubes containing the indicated amounts of puromycin and the tubes were incubated (37°) in an almost horizontal position (the liquid extended about 4 cm from the bottom of the tube). After 72 hr, dead cells were discarded by aspiration of the culture fluid, the attached cells were washed with 1 ml of growth medium, and growth was then estimated by counting the attached cells after detachment into fresh medium.

TABLE 2*

EFFECT OF PUROMYCIN ON NONVIABLE PARENT AND MUTANT CELLS

Puromycin, μg per ml	Culture			
	Pms^{-1}	*Pms*$^{-2}$	*Pmr*$^{-1}$	*Pmr*$^{-2}$
	Cell Number in Thousands			
0	460	520	460	500
4	11	380	...	...
8	3	80	420	...
12	...	2	300	...
16	...	...	240	520
24	...	...	31	380
40	...	...	...	430
80	...	...	...	17

* Each Petri dish received 4 ml of growth medium containing 5 × 10^6 cells obtained from suspended cultures. After 16 hr, the glass-attached cells were washed 3 times with 2-ml aliquots of a NaCl solution (0.15 *M*) and the washed cells were treated with 2 ml of a solution of nitrogen mustard (5 × 10^{-4} *M* in 0.15 *M* NaCl). The alkylating agent was destroyed after 5 min by the addition of growth medium (1 ml), and the cells were washed twice. Puromycin as indicated and growth medium were added to a final volume of 4 ml. The effect of the antibiotic was estimated after incubation (37°, 72 hr, atmosphere = 5 per cent CO_2 − air) by enumerating attached cells after the cells floating in the culture fluid were discarded.

The danger of contamination with mammalian cells from another culture, recently emphasized by the observations of Rothfels *et al.*,[5] was recognized. During these studies, in addition to the considerable care used in handling cultures, no fibroblast-like cell other than AMK 2-2 was maintained, and cultures were often recloned.

Cell counts were made with Levy counting chambers.

Puromycin.—Selection of mutants: To obtain cells resistant to puromycin, cultures of the susceptible parent were prepared on the surfaces of bottles (surface area = 75 cm^2). After heavy, but less than maximal growth was reached (approximately 4×10^6 cells), puromycin (8 μg per ml) was added to the growth medium. When about 95 per cent of the cells had been killed (about 24 hr), the medium was replaced with fresh growth medium lacking puromycin and the culture

TABLE 3*

THE PRESENCE OF Pm^{r-1} CELLS IN Pm^{s-2} CULTURES

Culture			Puromycin,	
Pm^{s-2}† (Approx. Number of Cells Added)	Pm^{r-1}	Pm^{r-2}	μg per ml	Number of Colonies per Dish
100	...	...	...	95, 81; 135, 112
100	...	...	4	90, 92; 139, 130
100	...	...	8	0, 0; 0, 0
10,000	...	...	12	0, 0; 0, 0
10,000	...	...	40	0, 0; 0, 0
100,000	...	...	12	0, 1; 0, 2
100,000	...	...	40	0, 0; 0, 0
300,000	...	...	12	1, 2; 5, 3
300,000	...	...	40	0, 0; 0, 0
...	100	...	...	95, 130; 124, 111
...	100	...	12	116, 97; 82, 99
100,000	100	...	12	107, 107; 93, 131
300,000	100	...	12	102, 108; 107, 125
...	...	100	...	109, 111; 86, 96
...	...	100	40	98,119; 98, 88
100,000	...	100	40	114, 136; 112, 84
300,000	...	100	40	97, 154; 74, 90

* The culture mixtures in Petri dishes contained cloning medium and the indicated cell number and puromycin concentration in a final volume of 4 ml. On the 4th and again on the 8th day of incubation (37°, atmosphere = 5 per cent CO_2 – air), the medium was replaced with fresh medium containing the same concentration of puromycin as initially. After a total incubation period of 11 or 12 days, the colonies were fixed and stained[9] and enumerated. The semicolons separate the results of experiments with two different recloned cultures.

† The Pm^{s-2} cultures were obtained by cloning the original single clone isolate and the experiment was made as soon as the recloned (suspended) cultures attained sufficient cell density (about 3×10^5 cells per ml).

was grown to its original density. The cyclic process of growth and selection was repeated (6 cycles) until some of the cultures were no longer affected by the toxic agent. The resistant cultures (designated Pm^{r-1}) were capable of growth in the presence of 12, but not 40 μg per ml of the antibiotic.

Cells resistant to higher levels (Pm^{r-2}) were selected from the less resistant mutant cultures with growth medium containing 40 μg of puromycin per ml. In contrast to the less resistant mutants, the Pm^{r-2} cells were obtained with ease, after a single cycle of selection and regrowth. Attempts to obtain cells more resistant than the Pm^{r-2} ones were unsuccessful.

The resistant mutants were cloned and their descendants were maintained in suspended cultures in the absence of puromycin.

Heterogeneity of parent culture: Examination of the descendants of parent (wild type) cells cloned in the absence of puromycin revealed two major classes, a highly sensitive one, designated Pm^{s-1}, and a less sensitive one, designated Pm^{s-2}. The behavior of the parent and mutant clonal isolates in the presence of various con-

centrations of the antibiotic is illustrated in Table 1. The mutant cultures studied had been cultivated in the absence of puromycin for more than 100 generations.

Response of nonviable cells: Parent and mutant cells made nonviable (incapable of cell division) by treatment with nitrogen mustard behaved in a similar manner to the respective growing cells (Table 2). The effect of puromycin was to cause the detachment of the cells from the glass surface. The time of addition of the antibiotic was not important, identical results were obtained when puromycin was added 0, 1, 2, or 3 days after treatment of the cells with the alkylating agent.

Evidence for sequential mutations: The isolation of four heritably different cultures with gradually increasing resistance coupled with the ease with which the Pm^{r-2} cells were obtained from the less resistant ones suggested that the origin of the most resistant cells might involve sequential mutational events rather than a single genetic change. Results consistent with a mechanism involving sequential mutations have been obtained by the quantitative examination of cultures for cells with increased resistance.

Of the ten recloned Pm^{s-2} cultures tested, four were found to contain small numbers of cells with resistance levels similar to the Pm^{r-1} cultures, but none contained detectable Pm^{r-2} cells. The results obtained with two of the cultures are shown in Table 3. With Pm^{r-1} cells, on the other hand, each of the recloned cultures studied yielded relatively large numbers of Pm^{r-2} cells. The results of one of the experiments are shown in Table 4.

TABLE 4*

THE PRESENCE OF Pm^{r-2} CELLS IN Pm^{r-1} CULTURES

Culture		Puromycin, μg per ml	Number of Colonies per Dish, Pm^{r-1}
Pm^{r-1} (Approx. Number Cells Added)	Pm^{r-2}		
100	...	...	126, 83
100	...	8	111, 111
100	...	12	84, 97
100	...	16	37, 40
100	...	24	0, 0
100	...	40	0, 0, 0, 0
1,000	...	40	0, 0, 3, 3
10,000	...	40	15, 30, 16, 11
100,000	...	40	199, 169, 148, 193
...	100	...	91, 109
...	100	40	85, 68
1,000	100	40	95, 112
10,000	100	40	113, 80
100,000	100	40	202, 267

* The culture mixtures were prepared and treated as described for Table 3. The Pm^{r-1} and Pm^{r-2} cultures had been recloned and used as described for the Pm^{s-2} cultures of Table 3.

The possibility was considered that an occasional colony can be formed by genotypically susceptible cells capable, for unknown reasons, of growth for several generations in an otherwise lethal concentration of puromycin. To test this, the cells of some of the colonies considered to be resistant were evenly dispersed in fresh medium containing puromycin. Their resistance was evidenced (after 6 days) by the formation of large numbers of new colonies. In addition, some of the colonies were cultured in the presence of puromycin for an additional period of time (4–8 days) and the cells were then transferred to test tubes. When sufficient growth had occurred, resistance levels were tested in titration experiments as

TABLE 5*

EFFECT OF 8-AZAGUANINE ON THE GROWTH OF PARENT AND MUTANT CELLS

8-Azaguanine, μg per ml	Culture		
	Pm^{s-1}†	Pm^{r-1}	Ag^r
	Cell Number in Thousands		
0	97	61	75
0.10	84	...	...
0.33	0	1	...
1.0	...	...	78
3.3	...	...	51
10	...	...	38
33	...	...	26
100	...	...	13
300	...	...	8

* The cells were from suspended cultures in the exponential phase of growth. One-milliliter aliquots of suspensions in growth medium (5,000 cells per ml) were added to test tubes containing the indicated amounts of 8-azaguanine. After incubation (37°) for 4.5 days, growth was estimated by counting the attached cells after detachment with a rubber spatula.

† The Pm^{s-1} and Pm^{r-2} cultures behaved identically with the susceptible cultures shown.

described for Table 1. The cells derived from Pm^{s-2} and Pm^{r-1} cultures were indistinguishable from the Pm^{r-1} and Pm^{r-2} mutants, respectively.

8-Azaguanine.—Selection of resistant mutant: Resistant cells were selected from susceptible parent cultures by repeated exposure to azaguanine (1 μg per ml) and regrowth in its absence as described for the Pm^{r-1} mutant. The culture (designated Ag^r) has been grown for more than 50 generations in the absence of the purine analogue without detectable loss in resistance.

"One-step" increase in resistance: In striking contrast to the relatively small and graded increases in resistance observed with puromycin, as shown in Table 5, the cells selected in the presence of 1 μg per ml of azaguanine grew, albeit slowly, in a medium containing a 100 times greater concentration of the analogue.

The results of experiments with single cells (from the uncloned culture not previously exposed to concentrations of the analogue greater than 1 μg per ml) also indicated a "one-step" increase in resistance of the Ag^r cells; no evidence for cells with intermediate levels of resistance was found. Thus, when 100 cells were plated (growth medium supplemented with 5 per cent horse serum) with 0, 3, 10, 30, and 100 μg per ml of analogue, the number of colonies formed was 59, 51, 55, 61, and 54, respectively.

Discussion.—While cultured mammalian cells cannot be handled in numbers as large as those conveniently manipulated by the microbe geneticist, it is indeed encouraging to find that a few mutant cells can be selected from at least a relatively large population.

The graded increase in resistance observed with puromycin was a not wholly unexpected result in view of the similar observations made with drug-resistant bacteria[6] and with metabolic analogue-resistant neoplastic cells cultured in mice.[10, 11] Although it seemed possible that a study of the puromycin-resistant cultures would reveal another similarity with the bacterial system,[12, 13] the existence of cells with many degrees of resistance intermediate between the most susceptible and the most resistant ones, this does not appear to be the case.

Cultures derived from single susceptible cells commonly contain small numbers of low resistance mutants but no detectable high resistance ones. Coupled with the presence of large numbers of high resistance mutants in all clonal descendants of lower resistance ones, these observations are consistent with a mechanism involving

sequential mutations to puromycin resistance. The possibility is obviously not excluded that susceptible cells may, in addition, give rise to highly resistant ones by a single mutational event but at a rate too low to be detected by present methods.

Summary.—Cells with heritable resistance to the antibiotic puromycin or the purine analogue 8-azaguanine have been isolated from a culture of fibroblast-like mammalian cells, AMK 2-2. Resistance to puromycin, but not to azaguanine occurs in relatively small steps. Evidence has been presented to show that resistance to the former compound involves sequential mutational events.

The technical assistance of Miss Marcia Heasley is gratefully acknowledged.

* This investigation was supported by a research grant from the National Institutes of Health, United States Public Health Service.

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*ESTIMATION OF MUTATION RATES WITH MAMMALIAN CELLS IN CULTURE**

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Communicated by Curt Stern, April 3, 1959

In a previous report[1] the isolation and some of the properties of cultured mammalian cells heritably resistant to metabolic analogues were described. With one of the toxic agents, puromycin, low resistance (Pm^{r-1}) and higher resistance (Pm^{r-2}) mutants were obtained. Evidence was presented to show that the increase in resistance from the parent (Pm^{s}) to the high resistance mutant involves at least two sequential mutational events.

To determine the rates of these events the classical method of Luria and Delbrück[2]

was used. The mutation rates from $Pm^s \rightarrow Pm^{r-1}$ and from $Pm^{r-1} \rightarrow Pm^{r-2}$ have been estimated to be approximately 4×10^{-6} and 1×10^{-4} mutations per cell per generation, respectively.

The purpose of this report is to detail the procedures used and to show the evidence for the validity of the mutation rate measurements.

Materials and Methods.—The exact origin of the cultured mammalian cell used in this work, AMK 2-2, is in doubt, but recent studies[3] have suggested its derivation from Earle's mouse fibroblast strain L.[4] Other materials and culture methods have been previously described.[1]

Experiments to estimate mutation rates were initiated by inoculating each of 25 to 50 test tubes with 100 cells in 2 ml of cloning medium (basal medium enriched with 15 per cent human and 5 per cent horse serum). When sufficient growth had occurred (9–15 days), the cells were detached with a rubber spatula and dispersed by vigorous aspiration. The suspended cells from 4 or 5 tubes, chosen at random, were enumerated in a Levy counting chamber and the average was taken as the number of cells per tube. Aliquots of the cultures were then added to Petri dishes (60 mm) containing puromycin and cloning medium to a final volume of 4 ml. To add the antibiotic with greater accuracy it was mixed with the cloning medium and dispensed with it.

On the 4th and again on the 8th day of incubation (37°, atmosphere = 5 per cent CO_2-air), the medium was replaced with fresh medium containing the same concentration of puromycin as initially. After an additional 4 days, the colonies were enumerated following fixation and staining.

Reliability of the Plating Method.—To determine whether plating of the AMK cells yields fluctuations larger than those ascribable to random sampling, parallel platings were made from single cultures both in the presence and absence of puromycin (Table 1). As can be seen from the table, the agreements obtained between

TABLE 1

PARALLEL PLATINGS FROM THE SAME CULTURE

Sample No.	Experiment No. 27A	28A	29A	9B	9B	25B
1	11	11	6	3	1	12
2	11	11	6	8	2	18
3	9	15	2	7	2	20
4	11	8	11	3	1	13
5	14	14	8	5	5	14
6	6	10	6	5	4	24
7	8	9	9	4	4	12
8	11	7	1	6	5	18
9	12	7		10	3	19
10	9	11		6	3	17
11	13	11		6	0	...
12	9	10		10	4	...
13	14	15	...	...	...	...
Cell culture	Pm^{r-1}	Pm^{r-1}	Pm^{r-1}	Pm^{s*}	Pm^s	Pm^{r-1}
Number of cells plated	10	10	5	1×10^5	1×10^5	4,800
Puromycin, μg per ml	...	...	...	12	12	40
Mean	10.6	10.7	6.1	6.1	2.8	16.7
Variance	5.6	7.2	11.2	5.5	2.7	15.3
χ^2	6.3	7.5	12.9	9.9	10.6	8.2
P	0.9	0.8	0.08	0.5	0.5	0.5

* The designation Pm^s will be used to describe Pm^{s-2} cultures (see reference 1).

TABLE 2

RESISTANT COLONIES (Pm^{r-1}) FROM SIMILAR CULTURES OF PARENT CELLS

Experiment No. →	1	4		5		7
Aliquot Plated, ml →	0.8	0.3	0.9	0.15	1.5	1.0
Culture No.	Number of Resistant Colonies					
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	21
4	0	0	0	0	0	0
5	0	0	0	24	171	4
6	0	0	1	0	0	0
7	0	0	0	0	0	0
8	1	0	0	0	1	0
9	3	2	4	3	2	0
10	0	11	42	0	0	0
11	0	0	0	0	0	0
12	0	2	10	0	0	0
13	0	0	0	0	0	0
14	0	...	...	0	0	0
15	0	...	...	0	0	0
16	2	...	...	0	0	...
17	0	...	...	0	0	...
18	0	...	...	0	0	...
19	0	...	...	0	2	...
20	18	...	...	0	0	...
21	0	...	...	1	10	...
22	0	...	...	0	0	...
23	0	...	...	0	0	...
24	0	...	...	0	0	...
Average per sample	1.0	1.2	4.5	1.2	7.8	1.6
Variance (corrected for sampling)	12.7	...	131	...	1,206	26.3
Average per culture	2.5	7.7	9.9	15.5	10.3	3.1
Cells per culture	1.0×10^6	6.7×10^5		4.0×10^5		3.3×10^5
Puromycin, μg per ml	12.0	12.0		11.4		8.0
Mutation rate*	0.8×10^{-6}	4.0×10^{-6}		6.2×10^{-6}		3.3×10^{-6}
Standard deviation / Average: found	3.6	2.5		4.5		3.2
Standard deviation / Average: calc.	1.7	1.0		1.2		1.4

* Mutations per cell per generation.

the variance and the mean indicate Poisson distributions, confirmed by application of the χ^2 test. Hence, it is unlikely that large errors of unknown origin occurred.

Mutation Rate of Parent Cells ($Pm^s \rightarrow Pm^{r-1}$).—The number of cells capable of producing colonies in the presence of 8–12 μg per ml of puromycin was measured in a series of similar cultures as described under "Materials and Methods" (Table 2). Mutation rates were calculated with equation 8 of Luria and Delbrück.[2] As can be seen from the table, reasonable agreement was obtained in the four experiments, the average value being 3.6×10^{-6} mutations per cell per generation. Also shown are the ratios of the standard deviations to the average numbers of resistant colonies. The similarities of the calculated and experimentally determined ratios are in agreement with the assumption that the mutational events occurred in the absence of puromycin. It is also of interest to note that the exact concentration of puromycin did not appear to affect the calculated mutation rates.

Mutation Rate from Low to High Resistance ($Pm^{r-1} \rightarrow Pm^{r-2}$).—Estimation of the high resistance mutants in similar cultures of the low resistance ones was carried out as with the parent cells (Table 3). The mutation rates were calculated by the "median" method of Lea and Coulson,[5] the average value being 0.8×10^{-4} mutations per cell per generation.

Control Experiments.—The fluctuations of the numbers of resistant cells as shown

in Tables 2 and 3 were much higher than can be accounted for by sampling errors (Table 1). The question arises, however, what factors in addition to mutation and growth of mutants may have contributed to the fluctuations observed. The following possibilities were considered.

(1) *Some of the culture tubes initially received one or more resistant cells:* This possibility, seriously considered for the studies with Pm^{r-1} cells, cannot be excluded. With three experiments (including experiment 11, Table 3), however, examination of the Pm^{r-1} cells (20 dishes, 100 cells per dish) used to prepare the series of similar cultures revealed no Pm^{r-2} cells.

(2) *The extent of growth in the culture tubes was highly variable:* Before use, culture tubes were screened for acid production and macroscopically for growth. Cell numbers were then determined in 4 or 5 of the remaining tubes chosen at random. Batches of cultures with differences greater than ±15 per cent, rarely encountered, were discarded.

(3) *Manipulation resulted in depression of viable cell numbers in some but not all culture tubes:* Detachment of the cells from the culture tubes and vigorous aspiration of the suspension had no detectable effect on viability. Thus, when 20 cells

TABLE 3

HIGH RESISTANCE COLONIES (Pm^{r-2}) FROM SIMILAR CULTURES OF LOW RESISTANCE CELLS (Pm^{r-1})

Experiment No.→	1	2		4		11
Aliquot plated, ml→	1.5	0.3	1.0	0.25	0.75	0.65
Culture No.	Number of Resistant Colonies					
1	4	7	9	5	16	22
2	1	18	37	18	33	17
3	53	10	20	2	8	13
4	1	2	3	17	73	1
5	14	9	29	2	10	6
6	0	72	207	5	12	267
7	1	3	0	6	39	12
8	0	3	3	1	9	8
9	3	4	38	0	0	23
10	6	1	3	24	108	15
11	4	21	89	24	72	22
12	45	8	8	2	17	2
13	18	4	5	28	99	45
14	4	6	7	8	28	2
15	15	4	24	5	5	100
16	5	7	20	11	62	7
17	2	4	11	0	3	21
18	0	6	20	5	20	9
19	48	3	2	12	42	...
20	12	3	8	2	18	...
21	0	0	7	2	5	...
22	16	...	...	34	124	...
23	1	...	...	1	9	...
24	0	...	...	4	11	...
25	11	...	...	...	...	...
Average per sample	10.6	9.3	26.2	9.1	34.3	32.9
Variance (corrected for sampling)	229	...	2088	...	1272	3899
Average per culture	14.1	62.0	52.4	72.8	91.6	101
Cells per culture	20,000	48,000		88,000		163,000
Puromycin, μg/ml	40	40		40		38
Mutation rate*	0.9×10^{-4}	0.9×10^{-4}		1.0×10^{-4}		0.5×10^{-4}
Standard deviation / Average (found)	1.4	1.7		1.0		1.9
Standard deviation / Average (calc.)	1.3	1.0		0.9		0.9

* Mutations per cell per generation.

TABLE 4

EFFECT OF COMBINING SAMPLES FROM PAIRS OF CULTURES*

Culture No.	Number of Resistant Colonies: Individual Cultures	Combined Cultures: Found	Combined Cultures: Calculated
1	4	2	3
2	2		
3	0	16	21
4	41		
5	15	4	8
6	0		
7	2	11	8
8	14		
9	1	0	1
10	0		
11	2	1	2
12	2		
13	4	8	12
14	20		
15	3	11	11
16	18		
17	1	4	1
18	0		
19	0	0	2
20	3		
21	59	37	32
22	2		
23	0	18	11
24	21		

* The cultures (2 ml), initiated with 100 Pm^{r-1} cells, were allowed to grow to 30,000 cells per tube. Plated were 1 ml aliquots of the suspended cells from each tube (individual cultures) and 1.0 ml aliquots of equal mixtures (combined cultures) prepared as indicated. The puromycin concentration was 40 μg per ml.

from cultures 7, 9, 11, and 13 were plated after use in experiment 4 (Table 3), 22, 19, 22, and 17 colonies were formed, respectively.

(4) *Differences in growth rate:* Doubling times of parent and mutant clonal isolates in the absence of puromycin were found to be indistinguishable, about 24 hours. The growth rate of resistant cells in mixtures containing relatively large numbers of more susceptible cells has, however, not been studied.

(5) *Unrecognized variables in plating efficiency with puromycin:* The number of colonies formed in the presence of puromycin appears to be a valid measure of the number of resistant cells present in the inoculum. In evidence is the acceptable proportionality obtained when different numbers of cells are plated from the same test cultures (experiments 4 and 5, Table 2; experiments 2 and 4, Table 3).

Further support was obtained by estimating resistant cells in mixtures of suspensions from pairs of culture tubes (Table 4). As can be seen from the table, the numbers of colonies yielded by the combined cultures were similar to the calculated values.

Discussion and Summary.—The availability of mutant mammalian cells in culture, manipulatable by procedures similar to those used with other microorganisms, has made possible the estimation of two mutation rates by the method of Luria and Delbrück.[2] The heritable changes studied, the acquisition of resistance to the antibiotic, puromycin, and the conversion of these mutants to ones with greater resistance, were calculated to occur at rates of approximately 4×10^{-6} and 1×10^{-4} mutations per cell per generation, respectively. It will be of the greatest interest

to determine whether these mutational processes can be used to study mutagenesis and antimutagenesis.[6]

The Luria-Delbrück analysis provides additional information, of course, not directly concerned with the rate of mutation. From the results obtained it may be concluded that the heritable increases in puromycin resistance are not dependent upon the presence of the antibiotic. Thus, cellular changes not involving mutation *per se*, like those encountered with bacteria[7,8] and protozoa,[9] may be ruled out to explain the origin of the puromycin-resistant cells. It should be noted that the work of Law[10] with A-methoperin resistant leukemic cells in mice led him to the same conclusions.

The question arises whether the changes studied result from genic or chromosomal alterations. With cultured mammalian cells, which commonly undergo chromosomal alterations,[11] the possibility of chromosomal rather than intragenic change is attractive. Unfortunately, methods which could supply at least presumptive evidence[12] on this point cannot yet be applied to cultured mammalian cells.

The authors gratefully acknowledge the numerous suggestions and encouragement of Drs. Milton Weiner and François Lamy and the technical assistance of Miss Marcia Heasley.

* This investigation was supported by a research grant from the National Institutes of Health, United States Public Health Service.

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*AN INFECTIOUS RIBONUCLEIC ACID DERIVED FROM INFLUENZA-INFECTED CELLS**

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Communicated by Thomas Francis, Jr., April 13, 1959

Several workers have recently reported the preparation of infectious ribonucleic acid from virus-infected cells of fluids of tissue cultures infected with animal viruses (poliovirus, mengo encephalitis, encephalomyocarditis, foot-and-mouth disease), using the phenol method described by Gierer and Schramm[1] for TMV. We have

found in our present studies that the treatment by phenol of tissue infected with influenza virus, yielded an infectious fraction which was inactivated by RNA-ase. We were unable to obtain, however, any infectious fraction from phenol extracts of purified concentrates of influenza virus. The results obtained suggest that the infectious fraction found in the aqueous phase of phenol extracts of infected cells possesses many properties which distinguish it from the intact virus.

In a typical experiment, a suspended culture of 14-day old chorioallantoic membranes was inoculated with a massive dose ($10^{8.0}$ EID_{50}) of mouse adapted Asian strain of influenza A virus. The infecting dose was about 100 EID_{50} per cell. After one hour of incubation at 37°C the tissue was washed to remove virus bound superficially to the tissue. The membranes were then placed into fresh culture fluid which contained no virus. By the 18th hour when maximal virus growth was evident, the chorioallantoic membranes (CAM) were rapidly frozen, ground to fine powder and mixed with the infected culture fluid. The suspension was centrifuged and the supernate removed and extracted with 90 per cent water-saturated phenol at 4°C. After various manipulations, an aqueous phase free of phenol was obtained and designated as the undiluted RNA preparation of influenza virus-infected cells.

TABLE 1

THE HOST RANGE OF TWO PREPARATIONS OF INFLUENZA VIRUS

Host	Preparation	Infectivity* MLD_{50}	Infectivity* EID_{50}	Infectivity* TCD_{50}
Chick kidney cells	Intact virus	...	...	4.5
	RNA	...	...	2.0†
Mouse lung	Intact virus	4.0	...	...
	RNA	0	...	...
Amniotic cavity	Intact virus	...	7.5	...
	RNA	...	0	...

* Infectivity titer expressed as the negative log of the dilution which will infect 50 per cent of the given host.

† The titer as determined by visual cytopathology and the hemadsorption reaction using 0.4 per cent fowl red blood cells.

TABLE 2

COMPARISON OF PROPERTIES OF INFECTIOUS RIBONUCLEIC ACID AND INFLUENZA A VIRUS

Treatment	Infectivity Titer,‡ $TCID_{50}$ per ml Intact Virus	Infectivity Titer,‡ $TCID_{50}$ per ml RNA Fraction
Control	4.8	1.8
After RNA-ase treatment*	4.5	0
After 3 hrs. at 37°C.	4.8	0
Normal ferret serum 30 min at room temperature†	4.5	0.5
Pooled human gamma globulin 30 min at room temperature†	0	2.0
Sediment from ultracentrification	5.3	No sediment
Supernate from ultracentrification	1.3	1.8
Sediment from precipitation with 1 *M* NaCl (3× washed)	2.0	1.5

* The RNA-ase treatment for 2 hr at 20°C.

† The normal ferret serum and the pooled human gamma globulin were diluted one to ten prior to incubation with the two preparations.

‡ All titers are expressed as the negative log of the dilution which will infect 50 per cent of inoculated chick kidney culture tubes or produce a positive hemadsorption reaction.

The infectivity of the phenol extracted material could be demonstrated only in chick kidney culture and not in the amniotic cavity of embryonate eggs or in mice as shown in Table 1. The interpretation of such phenomenon must await additional studies now in progress. There is, however, suggestive evidence that the amniotic cavity or the mouse lung contains sufficient inhibitory substances to inactivate the infectivity of the RNA preparation.

The infectious component in the RNA preparation is endowed with many properties which differentiate it from the intact virus. Table 2 shows a comparison between the properties of the intact virus and the RNA preparation with respect to RNA-ase, temperature, normal and immune serum, ultracentrifugation and 1 *M* NaCl solution. The results are in agreement with data presented by Colter *et al.*[2] and Alexander *et al.*[3] on poliovirus and rule out the possibility that the infective material present in the phenol extracts is likely to be residual intact virus.

It would be appropriate to mention now that the RNA-ase concentration used (25 micrograms per ml) did not have any effect on the intact virus or the host cell.

Le Clerc[4] has shown that RNA-ase does not act on the influenza virus nor does the enzyme inhibit amino acid incorporation in the host cells. RNA-ase stops the growth of the virus when it is added shortly after infection and it inhibits the so-called "dark phase" of virus multiplication. In our experience there was a slight inhibition when influenza virus was titrated with RNA-ase in the chick kidney cells.

TABLE 3

EFFECT OF DILUENTS ON INFECTIVITY OF RIBONUCLEIC ACID PREPARATION FROM INFLUENZA VIRUS

Diluents*	Dilution†	Cytopathic Effect‡
Hank's balanced salt solution	1:10	0
	1:100	0
(2–5)× conc. of Hank's balanced salt solution	1:10	0
	1:100	0
1 *M* NaCl + 0.4 *M* phosphate	1:10	+
	1:100	0
Mixture 199	1:10	+
	1:100	0
Eagle's basal solution	1:10	+
	1:100	0
2× conc. of Eagle's basal solution	1:10	+
	1:100	+

* In all instances, the chick kidney cells were exposed for 1 hr at 37°C to the RNA preparation, before addition of the nutrient fluid overlay.
† Six cultures of chick cells were used per dilution.
‡ The cytopathic effect was evident at the 3rd day of incubation and was always preceded by a positive hemadsorption reaction.

The infectivity of the virus in the presence of RNA-ase was of the order of $10^{3.7}$ $TCID_{50/ml}$ while the control had a titer of $10^{4.3}$, a difference of 0.6 of a log. These experiments strongly indicate that the action of RNA-ase is to interfere with the integrity of RNA and its action is not on the intact particle or the host cells.

The infectivity of the RNA preparations was shown to be of low order of magnitude, about 0.2 per cent that of the virus suspension from which they were prepared. It was found however, that the efficiency of infection of the RNA preparation can be increased to 0.4 per cent by use of the proper diluent. Results in Table 3 show that there is an increase in titer when the infectious RNA is diluted in a double concentration of Eagle's basal solution.[5] The increase in infectivity attained when RNA preparation of poliovirus is diluted with hypertonic salt solution, Alexander *et al.*,[3] is not found with RNA preparations made from cells infected with influenza virus.

For successful isolation of the infectious component of influenza virus, the infected tissue must be extracted with phenol at the peak of virus production. The results presented in Table 4 show the titer of the intact virus and the aqueous phase of the phenol extract at various intervals of the growth cycle. It appears that at the

TABLE 4

THE RELATION OF AGE OF INFECTION TO TITER OF INTACT VIRUS AND RIBONUCLEIC ACID PREPARATIONS

Elapsed Time, hr of Infection before Extraction	Intact Virus		RNA,
	Infectivity (EID_{50})*	Hemagglutination†	Infectivity* ($TCID_{50}$)
8	4.5	0	0
18	7.5	160	2.0
28	6.5	160	0.5
40	5.7	160	0

* Expressed as reciprocal logarithm of dilution of infectivity titer per ml. in the given host.
† Hemagglutination titers were obtained with 0.5 per cent suspension of fowl red blood cells.

TABLE 5

THE INFLUENCE OF TIME ON THE INFECTIVITY FOR TISSUE CULTURES OF VARIOUS PREPARATIONS OF INFLUENZA VIRUS

Preparations*	Infectivity Titer† ($TCID_{50}$) at the 4th day — Time, hr, of Incubation before Inoculation				
	1	3	7	12	18
Intact virus	4.5	4.7	4.5	4.3	4.3
Intact virus + RNA-ase‡	4.3	4.5	4.5	4.0	4.0
RNA	2.0	1.8	0.5	0	0
RNA + viral protein	1.8	2.0	1.8	1.8	1.5
RNA + viral protein + RNA-ase	0.5	0	0	0	0
RNA + protein of infected cells	1.8	1.5	1.5	1.8	1.5
RNA + protein of normal cells	1.8	1.5	1.0	0	0
Viral protein	0	0	0	0	0
Protein of normal cell	0	0	0	0	0

* The preparations were left standing for the stated periods at 7°C before inoculation into the cell cultures.
† Negative log to base 10 of the dilution which will infect 50 per cent of cultures in 2× Eagle's basal solution.
‡ RNA-ase final concentration 25 micrograms per ml.

eighth hour while the intact virus had a titer of $10^{4.5}$ EID_{50} per ml, the RNA preparation did not have any infectivity. This could be a reflection of the low concentration of viral components of the infected cells. However, at the 18th hour, the peak of virus titer is reached and it is at this period that the RNA preparations show the highest degree of infectivity. With prolonged incubation of the infected tissue, virus inactivation is evident by the decrease in the ratio $\frac{EID_{50}}{HA}$ resulting in a reduction of more than 30-fold in infectivity of the RNA preparation. This is in agreement with our failure to extract infectious RNA by phenol treatment of inactive preparations of influenza virus. In addition, our failure to extract infectious RNA from an active concentrated and purified preparation of influenza virus, led us to believe that the infectious RNA isolated from influenza-infected cells comes not from virus as such, but from components of the infected tissue formed during virus synthesis. This is in agreement with the work of Huppert and Sanders[6] in which they show that the infective material in phenol extract comes from a component in encephalomyocarditis-infected tissue and they were unable to show infectivity of the phenol extracts of a concentrated virus suspension.

Preliminary studies of the role of protein in increasing the efficiency of infection of the RNA preparation has shown that the protein fraction of the virus confers stability upon the infectious RNA and preserves the integrity of the infectious component over a long period of time as shown in Table 5. The method of Fraenkel-Conrat[7] was used to degrade influenza virus with acetic acid. It was possible to obtain native viral protein which is antigenic and possesses hemagglutinin activity. On

mixing the RNA and viral protein, infectivity could be demonstrated for a period of 18 hours, while the infectivity of the RNA preparation alone, starts decreasing after the 3rd hour. Specificity of protein action is quite apparent because on mixing the infectious RNA with protein isolated from uninfected cells, there is loss of infectivity of the RNA component after the 3rd hour as a result of progressive denaturation.

The progeny produced by the infectious ribonucleic acid (RNA) corresponded to the parent type strain with two main differences in biological properties: (1.) A marked divergence in infectivity titer in the chick kidney culture with the parent line being able to grow to a much higher titer, $10^{5.3}$ $TCID_{50}$ per ml (a difference of two log); (2.) a loss of ability to propagate in the allantoic cavity of embryonate eggs. However, inoculation into the amniotic cavity resulted in a titer of $10^{2.6}$ EID_{50} per ml.

Although this study is still in its infancy, it is hoped that the approach will considerably clarify the relationships existing between RNA and protein of different strains of influenza virus and will set the stage for a possible molecular interpretation of the antigenic shift of influenza virus.

* These studies were conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and were supported by the office of the Surgeon General, Department of the Army, Washington, D. C.

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*ON A SYMMETRY IN WEAK INTERACTIONS**

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Read before the Academy, April 29, 1959

1. Introduction.—The V-A theory of weak interactions[1] appears to be on an extraordinarily sound experimental footing in so far as the strangeness-conserving processes are concerned.[2] The decay process: $\mu \rightarrow e + \nu + \bar{\nu}$ leads uniquely to a V-A interaction for the physical fermions and, since no strong interactions are involved, to a V-A interaction for the "bare" fermions; unfortunately, it is not decided whether the charge-exchange or charge-retention order for the current is to be chosen since both orders lead to identical results for the V-A interaction. The nuclear beta decay experiments require a (V − 1.2 A) interaction in the charge-exchange order for the baryon and lepton currents separately; since the vector part of the interaction for nuclear beta decay is equal (within a few per cent) to the vector part of the interaction for muon decay, and since virtual pion effects can in principle explain the different coefficient in front of the axial vector part of the

interaction, it is indicated that the bare fermion interaction in nuclear beta decay is also V-A. Finally, the recent experimental findings[3] on the ratio of the processes: $\pi \to e + \nu$ to $\pi \to \mu + \nu$ argue in a striking fashion for the equality of the couplings of the $(\mu\nu)$ and $(e\nu)$ lepton pairs to the (np) baryon pair for the axial vector part of the interaction; since the pion renormalization effects cancel out, this equality must hold for the bare couplings. If we furthermore take cognizance of the fact that such processes as: $\mu \to 3e$, $\mu + p \to p + e$, and $\mu + n \to n + e$ are not observed, we can conclude that a satisfactory explanation of all strangeness-conserving weak processes would be obtained by postulating a universal V-A interaction among the three charge exchange pairs $(e\nu)$, $(\mu\nu)$, (np).

The situation regarding the strangeness-nonconserving processes is less clearcut both because the experimental data are much more meager and also because the theoretical ambiguities are greater. If one adds the strangeness-nonconserving baryon pair (Δp) to the other three charge-exchange pairs, one can in principle explain[4] both the leptonic and nonleptonic decay modes of the strange baryons (Λ, Σ, Ξ) and the strange meson (K). One can even argue that the total evidence from the strange particle decays is in favor of comparable amounts of V and A interaction for the bare fermions despite the uncertain status of the different renormalization corrections for the different processes. However, it is not clear why such strangeness-nonconserving charge-exchange baryon pairs as (Σ^0, p) and (Σ^-, n) should not be included and it is not even absolutely certain that the charge-exchange pair (Σ^+, n) should be excluded. One uncertain experimental point of particular importance is whether the $\Delta I = {}^1/_2$ selection rule first suggested by Gell-Mann holds for the nonleptonic decay modes of the strange particles. This isotopic spin selection rule requires an equal admixture of the charge-exchange four-fermion coupling (Λp)(np) and the charge-retention four-fermion coupling (Λn)(nn). The present experimental data are consistent with this selection rule but are also equally consistent with the $I = {}^1/_2$ strangeness nonconserving current rule proposed by other authors.[5] The arguments for using charge-exchange currents for the strangeness-nonconserving processes are therefore not as strong as for the strangeness-conserving processes.

In order to eliminate the theoretical ambiguity for the strangeness-nonconserving processes, we shall adopt the viewpoint that the (Λ, n, p) baryon triplet is the complete analogue of the (μ, e, ν) lepton triplet in so far as the weak interactions are concerned. Authors like Sakata[6] and Okun[7] have proposed specific models in which the Σ and Ξ hyperons and the π and K mesons are regarded as bound states of Λ, n, p, and their antiparticles. Our remarks will not depend on the choice of a specific model for the strong interactions.

2. Symmetry Principle between the Baryon and Lepton Triplets.—The (Λ, n, p) baryon triplet—in order of decreasing mass—appears to be the minimum number of strongly interacting particles which are necessary to explain conservation of charge, isotopic spin, and strangeness (in strong interactions). In weak interactions, conservation of isotopic spin and strangeness no longer hold and the baryon triplet (Λ, n, p) bears a striking similarity to the lepton triplet (μ, e, ν) in several respects. In the first place, the mass difference between the component of the isotopic doublet (n, p) is of the same order of the mass difference between the members of the pair (e, ν) and both are probably explicable in terms of electro-

magnetic self-energies. Secondly, the mass difference between the third member of the baryon triplet, Λ, and the isotopic doublet (n, p) is of the same order as the mass difference between μ and the (e, ν) pair; there seems to be a connection between the large mass of the muon and the role of strangeness in weak interactions. Pursuing this analogy, we would argue that the mass difference between Λ and the nucleon cannot be explained by any of the known self-energy effects, since the corresponding mass difference between muon and electron is apparently not due to any of these self-energy effects (strong, electromagnetic, or weak). Furthermore, we would not expect any leptons heavier than the muon since a baryon triplet suffices to account for the conservation laws in strong interaction; by the same token, it would not be surprising to find additional baryons and bosons (mesons) which are more complicated composite systems of the three fundamental baryons.

In order to give greater meaning to the above remarks and to suggest explicit experimental tests, we postulate the following symmetry principle: all weak interactions are invariant under the following simultaneous transformation:

$$\Lambda \rightleftarrows \mu, \qquad n \rightleftarrows e, \qquad p \rightleftarrows \nu \tag{A}$$

In this note, we investigate the consequences of the transformation (A). We shall find that (A) does not contradict any of the known facts and leads to some interesting predictions which can be tested.

We classify in Tables 1 and 2 all possible four-fermion interactions in accordance with principle (A); Table 1 lists the lepton-lepton interactions (and consequently also the baryon-baryon interactions) and Table 2 lists the baryon-lepton interactions (and consequently the lepton-baryon interactions). In these tables we have omitted all interactions which can be derived by permutations of the spinors i.e., we do not distinguish among $(\bar{\psi}_1\psi_2)(\bar{\psi}_3\psi_4)$, $(\bar{\psi}_1\psi_4)(\bar{\psi}_3\psi_2)$, $(\bar{\psi}_3\psi_2)(\bar{\psi}_1\psi_4)$, and $(\bar{\psi}_3\psi_4)(\bar{\psi}_1\psi_2)$. We have also indicated whether the weak process has been observed (favorable), could have been observed but has not been observed (unfavorable), or for which there is no evidence either way (unknown). Under remarks we have noted illustrations of weak processes which follow from the postulated four-fermion interactions.

3. Discussion.—The following comments can be made concerning Tables 1 and 2.

(1) The symmetry principle (A) reduces to 4 the number of four-fermion interactions required to explain all the observed weak processes; this is to be compared to the 6 originally required by the Gell-Mann-Dallaporta tetrahedron.

(2) The four-lepton interactions $(\bar{\mu}e)$ $(\bar{e}e)$ and $(\bar{\mu}e)$ $(\bar{\mu}\mu)$ are in the unfavorable class. The first interaction appears to be forbidden because the decay $\mu^- \rightarrow e^- + e^- + e^+$ is never observed. The second interaction $(\bar{\mu}e)$ $(\bar{\mu}\mu)$ must also be forbidden for the same reason because it gives rise to an effective $(\bar{\mu}e)$ $(\bar{e}e)$ due to the virtual electromagnetic interaction. An estimate shows that this effective $(\bar{\mu}e)$ $(\bar{e}e)$ is of the order of $(1/2\pi)$ $(e^2/4\pi)$ ln $[(\lambda/m\mu)^2 + 1]$ compared to the original interaction, where λ is the cutoff momentum. Even if λ is taken equal to the muon mass, the four-lepton interaction $(\bar{\mu}e)$ $(\bar{\mu}\mu)$ must be forbidden to explain the experimental upper limit on the decay $\mu \rightarrow 3e$. It perhaps should be remarked that the interactions $(\bar{\mu}e)$ $(\bar{\mu}\mu)$ and $(\bar{\mu}e)$ $(\bar{e}e)$ would give rise to the decay $\mu \rightarrow e + \gamma$ although this would be of order $(e^2/4\pi)^3$ and consequently very small. If we

TABLE 1

LEPTON AND BARYON INTERACTIONS

Type	Interaction	Remarks
Favorable	$(\bar{\mu}\nu)\ (\nu\bar{e}) \longleftrightarrow (\bar{\Lambda}p)\ (\bar{p}n)$	$\mu^- \to e^- + \nu + \bar{\nu}$ $\Lambda \to p + \pi^-$
Unfavorable	$(\bar{\mu}e)\ (\bar{e}e) \longleftrightarrow (\bar{\Lambda}n)\ (nn)$	$\mu^- \to e^- + e^- + e^+$ $\Delta I = {}^1/_2$ selection rule
	$(\bar{\mu}e)\ (\bar{\mu}\mu) \longleftrightarrow (\bar{\Lambda}n)(\bar{\Lambda}\Lambda)$	$\mu^- \to e^- + e^- + e^+$
	$(\bar{\mu}e)\ (\bar{\mu}e) \longleftrightarrow (\bar{\Lambda}n)\ (\bar{\Lambda}n)$	$e^- + e^- \to \mu^- + \mu^-$ $K_1^\circ - K_2^\circ$ mass difference
Unknown	$(\bar{\mu}\mu)\ (\bar{\mu}\mu) \longleftrightarrow (\bar{\Lambda}\Lambda)\ (\bar{\Lambda}\Lambda)$	
	$(\bar{\mu}\mu)\ (\bar{e}e) \longleftrightarrow (\bar{\Lambda}\Lambda)\ (\bar{n}n)$	
	$(\bar{\mu}\mu)\ (\bar{\nu}\nu) \longleftrightarrow (\bar{\Lambda}\Lambda)\ (\bar{p}p)$	
	$(\bar{e}e)\ (\bar{e}e) \longleftrightarrow (\bar{n}n)\ (nn)$	
	$(\bar{e}e)\ (\bar{\nu}\nu) \longleftrightarrow (\bar{n}n)\ (\bar{p}p)$	$e + \nu \to e + \nu$
	$(\bar{\nu}\nu)\ (\bar{\nu}\nu) \longleftrightarrow (\bar{p}p)\ (\bar{p}p)$	

TABLE 2

BARYON-LEPTON INTERACTIONS

Type	Interaction	Remarks
Favorable	$(\bar{p}n)\ (\bar{e}\nu) \longleftrightarrow (\bar{\nu}e)\ (\bar{n}p)$	$n \to p + e^- + \bar{\nu}$
	$(\bar{\Lambda}p)\ (\nu\mu) \longleftrightarrow (\bar{\mu}\nu)\ (\bar{p}\Lambda)$	$K^+ \to \mu^+ + \nu$
	$(\bar{p}n)\ (\bar{\mu}\nu) \longleftrightarrow (\nu e)\ (\bar{\Lambda}p)$	$\mu^- + p \to n + \nu$ $\Lambda \to p + e^- + \bar{\nu}$
Unfavorable	$(\bar{\Lambda}n)\ (\bar{\mu}e) \longleftrightarrow (\bar{\mu}e)\ (\bar{\Lambda}n)$	$K_2^\circ \to \mu^- + e^+$
	$(\bar{\Lambda}n)\ (\bar{e}\mu) \longleftrightarrow (\bar{e}\mu)\ (\bar{\Lambda}n)$	$K_2^\circ \to \mu^+ + e^-$
	$(nn)\ (\bar{\mu}e) \longleftrightarrow (\bar{e}e)\ (\bar{\Lambda}n)$	$\mu^- + n \to e^- + n$ $K_2^\circ \to e^- + e^+$
	$(\bar{p}p)\ (\bar{\mu}e) \longleftrightarrow (\bar{\nu}\nu)\ (\bar{\Lambda}n)$	$\mu^- + p \to e^- + p$ $K_2^\circ \to \pi^\circ + \nu + \bar{\nu}$
	$(\bar{\Lambda}\Lambda)\ (\bar{\mu}e) \longleftrightarrow (\bar{\mu}\mu)\ (\bar{\Lambda}n)$	$K_2^\circ \to \mu^+ + \mu^-$
Unknown	$(\bar{\Lambda}\Lambda)\ (\bar{\mu}\mu) \longleftrightarrow (\bar{\mu}\mu)\ (\bar{\Lambda}\Lambda)$	
	$(\bar{p}p)\ (\bar{\mu}\mu) \longleftrightarrow (\nu\nu)\ (\bar{\Lambda}\Lambda)$	
	$(\bar{p}p)\ (\bar{\nu}\nu) \longleftrightarrow (\bar{\nu}\nu)\ (\bar{p}p)$	$\nu + p \to \nu + p$
	$(nn)\ (\bar{\nu}\nu) \longleftrightarrow (\bar{e}e)\ (\bar{p}p)$	
	$(\bar{\Lambda}\Lambda)\ (\bar{e}e) \longleftrightarrow (\bar{\mu}\mu)\ (nn)$	
	$(\bar{n}n)\ (\bar{e}e) \longleftrightarrow (\bar{e}e)\ (nn)$	

accept these statements, our symmetry principle (A) now predicts that the four-baryon interactions $(\bar{\Lambda}n)\ (\bar{n}n)$ and $(\bar{\Lambda}n)\ (\bar{\Lambda}\Lambda)$ are forbidden. This implies that the only possible nonleptonic weak interaction which involves a half-integral change of isotopic spin is $(\bar{\Lambda}p)\ (\bar{p}n)$ and hence the $\Delta I = {}^1/_2$ selection rule of Gell-Mann cannot be valid. In general, the nonleptonic decays will involve a mixture of $\Delta I = {}^1/_2$ and $\Delta I = {}^3/_2$. More detailed discussion of this last point will be found in other papers.[4]

(3) We have placed the four lepton interaction $(\bar{\mu}e)\ (\bar{\mu}e)$ in the unfavorable class because its analogue (according to (A)), the four-baryon interaction $(\bar{\Lambda}n)\ (\bar{\Lambda}n)$, is unfavorable. The latter interaction gives rise to a so-called $\Delta S = 2$ transition and therefore yields too large a mass difference between K_1° and K_2° to be consistent with experiment.[8] Our statement concerning the forbiddenness of $(\bar{\mu}e)\ (\bar{\mu}e)$ could be checked in principle by looking for the process $e^- + e^- \to \mu^- + \mu^-$ but unfortunately the threshold energy for this reaction is 44 Bev for the laboratory electron. Another test would be to study the reaction $e^- + \mu^+ \to e^+ + \mu^-$ but the cross section is of the order of 10^{-45} cm^2.

4.—The self-conjugate baryon-lepton interactions $(\bar{\Lambda}n)\ (\bar{\mu}e)$ and $(\bar{\Lambda}n)\ (\bar{e}\mu)$ are unfavorable because the decay modes $K_2^\circ \to \mu^- + e^+$ and $K_2^\circ \to \mu^+ + e^-$ are unobserved.

5.—The baryon-lepton interactions $(\bar{n}n)$ $(\bar{\mu}e)$ and $(\bar{p}p)$ $(\bar{\mu}e)$ are unfavorable because the reactions $\mu^- + n \rightarrow e^- + n$ and $\mu^- + p \rightarrow e^- + p$ are not observed. Similarly, the baryon-lepton interaction $(\bar{\Lambda}\Lambda)$ $(\bar{\mu}e)$ is unfavorable because it will give rise to an effective $(\bar{n}n)$ $(\bar{\mu}e)$ or $(\bar{p}p)$ $(\bar{\mu}e)$ interaction via the strong interactions. This would imply, in accordance with our principle (A), that the baryon-lepton interactions $(\bar{\Lambda}n)$ $(\bar{e}e)$, $(\bar{\Lambda}n)$ $(\bar{\mu}\mu)$, and $(\bar{\Lambda}n)(\bar{\nu}\nu)$ are unfavorable. This is consistent with the experimental absence of the decays: $K_2^\circ \rightarrow e^- + e^+$, $K_2^\circ \rightarrow \mu^- + \mu^+$, and $K_2^\circ \rightarrow \pi^\circ + \nu + \bar{\nu}$.

6.—The remaining unknown four-lepton, four-baryon and baryon-lepton interactions represent weak scattering reactions with cross sections of the order of 10^{-45}cm^2. Experiments to decide whether these interactions are favorable or not will be very difficult.

We have found, therefore, that the symmetry principle (A) between the baryon triplet (Λ, n, p) and the lepton triplet (μ, e, ν) is completely consistent with existing experiments. Favorable interactions lead to favorable interactions and the negative is also true. The success of principle (A) makes it tempting to introduce formally the notion of isotopic spin and weak hypercharge for leptons. Assigning isotopic spin one-half for the (e, ν) pair and zero isotopic spin for the muon, the charge Q for any member of the baryon and lepton triplet can be written as:

$$Q = I_3 + (T + B - L)/2 \qquad \text{(B)}$$

This formula holds for both baryons and leptons if we assign $T = -1$ for the muon and $T = 0$ for the electron and neutrino, and if we identify T with the strangeness S in the case of the baryon. L (or B) is the lepton (or baryon) number and I_3 is the third component of the isotopic spin. If we adopt equation (B), the reaction $e^- + e^- \rightarrow \mu^- + \mu^-$, for example, would be forbidden because of the selection rule $\Delta T = 2$, which corresponds to $\Delta S = 2$.

* This work was supported in part by the Atomic Energy Commission.

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[2] Cf. Proceedings of the 1958 International Conference on High Energy Physics at CERN.

[3] Cf. review paper of Dr. C. S. Wu at the Gatlinburg Conference on "Weak Interactions," October, 1958 (to be published in *Rev. Mod. Phys.*).

[4] Cf. Okubo, S., R. E. Marshak, and E. C. G. Sudarshan, *Phys. Rev.*, **113**, 944 (1959).

[5] Cf. Okubo, Marshak, Sudarshan, Teutsch, and Weinberg, *Phys. Rev.*, **112**, 665 (1958).

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*CHANGES IN PLASMA 17-HYDROXYCORTICOSTEROIDS ACCOMPANYING SEXUAL MATURATION AND SPAWNING OF THE PACIFIC SALMON (ONCORHYNCHUS TSCHAWYTSCHA) AND RAINBOW TROUT (SALMO GAIRDNERII)**

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Communicated April 13, 1959

In the course of an investigation into the nature of the post-spawning death of the Pacific salmon,[1–3] marked hyperplasia of the adrenal cortical tissue was found to be present constantly in the sexually mature fish of both sexes.[4, 5] Beginning hyperplasia was observed in immature salmon taken on their spawning migration several months before they were due to spawn. This change became more pronounced with progressive development of the gonads. Studies on the anadromous form of the rainbow trout, the steelhead, which suffer a considerable mortality after spawning, also revealed the presence of marked hyperplasia of adrenal cortical tissue at full sexual maturity, whereas the nonmigratory rainbow trout (hatchery reared) which usually survive their initial reproductive effort showed very little hyperplasia of the adrenal tissue.

The present study was undertaken to determine the presence of adrenal corticoids in the blood plasma of salmon at various stages of their spawning migration and sexual development, as well as in fully mature rainbow trout, migratory and nonmigratory.

Material and Methods.—Source of fish: Twenty-two king salmon in their second, third, and fourth years of age were caught by hook and line in the sea in Monterey Bay in August, 1957, and just off the entrance of Bolinas Bay north of San Francisco in July, 1958. These fish migrate up the Sacramento and San Joaquin Rivers. Their gonads were in an infantile state, so that it was not possible to predict exactly how close they were to starting their spawning migration.

Fourteen male salmon were taken in wire traps on the Sacramento River in September, 1958, at a point designated as Fremont, about 125 miles from the sea. These were fall-run fish which began their fluvial migration from August to October with gonads already developing. With three exceptions they exhibited gonads in an advanced state of maturation and were probably due to spawn within 6 weeks to 2 months. Fourteen salmon were secured at Mill Creek, a tributary of the Sacramento River, approximately 285 miles up stream, in May, 1958. These were all spring run, i.e., those which leave the sea from March to May and, with two exceptions, their gonads were in a relatively early stage of development. The expectation was that they would not spawn for 4 to 5 months. They were taken in a trap at the top of a ladder on the Mill Creek Dam.

Spawning salmon were trapped at the Coleman Hatchery on Battle Creek, another branch of the Sacramento River, about 325 miles from the sea, October to December 1957, 1958. The Coleman salmon are fall-run fish. Some of these, after being artificially spawned, were held for 3 or 4 days before blood samples were drawn. Fifty ripe and spent (held) fish were studied.

Twelve king (Chinook) salmon were secured at the Rock Island Dam on the Columbia River some 500 miles up stream. These, all males in their second year (grilse), exhibited testes in a relatively early state of maturation.

Twenty-seven steelhead trout ready to spawn were caught in traps in the Eel River at two points 82 and 154 miles from the sea.† Seven sexually mature males and 25 immature nonmigratory rainbow trout were obtained from the hatchery of George Dufour, Santa Cruz, whom we wish to thank.

Bleeding: The fish were lightly anesthesized beforehand in a 1–25,000 solution of tricaine-

methanesulfonate (Sandoz) which was kept well aerated by means of an air pump and gas dispersers. Bleeding was performed by heart puncture using heparin as an anticoagulant. The blood sample was immediately put on ice and centrifuged. The plasma was drawn off and iced until the samples could be deep-frozen.

Determination of free plasma 17-hydroxycorticoids (17-OHCS): Plasma 17-OHCS were determined by the methods of Peterson and Wyngaarden, *et al.*[6] and Silber and Porter.[7] The Allen correction principle[8] for maximum absorption was applied.

Identification of the adrenal corticoids: The salmon plasma was extracted with methylene dichloride and chromatographed on paper, with standards in parallel, by Zaffaroni's method,[9] using chloroform saturated with formamide. The areas corresponding to hydrocortisone and cortisone and the area from cortisone to the front were eluted and rerun in the Bush (B_4) system.[10] Standards in each case were run in parallel.

Identification of the steroids were based on (*a*) photographs in ultraviolet light (λ 235.7 mμ), (*b*) reaction with "blue tetrazolium," (*c*) Rf values of substances compared with standards run in parallel, and (*d*) absorption spectra of sulfuric acid chromogens.[11]

RESULTS

Plasma Concentration of 17-Hydroxycorticosteroids.—(1) Sea salmon: Since only relatively small quantities of blood could be obtained from the fish caught by hook and line, it was necessary to pool the samples of plasma in order to obtain sufficient material for corticoid determination. Table 1 lists 17-OHCS values for two pools

TABLE 1

BLOOD LEVELS OF 17-HYDROXYCORTICOIDS (μG/100 ML) IN SEA SALMON

Date	Location	No. of Fish	Plasma
August, 1957	Monterey Bay	Pool of 4 fish	10.6
July, 1958	Bolinos Bay	Pool of 7 fish	13.2
July, 1958	Bolinos Bay	1 female	7.0

and a single fish, giving a mean concentration of 11.8 μg/100 ml. No distinction between sexes was made in the pools.[12]

(*2*) *Migrating salmon with maturing gonads:* Individual plasma corticoid determinations were made on 7 male and 7 female salmon secured at Mill Creek. Table 2 shows the results of the determinations. There was a very slight dif-

TABLE 2

BLOOD LEVELS OF 17-HYDROXYCORTICOIDS (μG/100 ML) IN SALMON ON SPAWNING MIGRATION

Fremont* Males	Mill Creek‡ Males	Mill Creek‡ Females
50.0	20.3	34.8
33.4	62.7	57.2
62.0	46.5	51.7
27.5	58.5	44.8
47.8	24.4	77.1
36.3	75.5	60.0
32.5	53.4	47.9
Mean 41.4 ± 12	49.0 ± 21	53.4 ± 13

* Fall run. ‡ Spring run.

ference between the Mill Creek males and females, namely 49.0 ± 21 μg/100 ml for the former and 53.4 ± 13 μg/100 ml for the latter. The Mill Creek males with immature testes exhibited a little higher 17-OHCS level than the Fremont males whose gonads were in an advanced stage of maturation: 49.0 ± 21 μg/100 ml and 41.4 ± 12 μg/100 ml, respectively. Twelve salmon (grilse) with immature

gonads taken at Rock Island Dam on the Columbia River had an average level of 89 μg/100 ml.

(3) *Spawning and spent (held) salmon:* Plasma 17-OHCS levels were determined in a total of 24 males and 26 females obtained at the Coleman Hatchery. Fourteen spawning and spent fish in 1957 had mean corticoid levels of 28.4 μg/100 ml for the males and 81.2 μg/100 ml for the females. Thirty-six fish in 1958 showed mean values of 32.4 ± 13 μg/100 ml for the males and 77.4 ± 28 μg/100 ml for the females. No significant differences were found between ripe and spent fishes.

TABLE 3

BLOOD LEVELS OF 17-HYDROXYCORTICOIDS (μG/100 ML) IN SPAWNING AND SPENT SALMON

1957		1958	
Males	Females	Males	Females
26.6*	113.0*	52.1	80.0
29.2*	47.4*	19.2†	59.1
42.1*	102.0*	37.1	48.0†
16.0*		37.1	50.9
		45.9	101.0
		13.5	62.6
		11.7	74.6
		46.6	83.1
		32.5	134.0
		11.7	54.7
		46.6	57.5
		32.5	69.3
		44.3	77.1
		14.6	74.5
		24.2	72.0
		37.5	59.1
		42.6	78.9
		27.8	158.0
Mean 29.0 ± 11	88.5 ± 35	32.4 ± 13	77.4 ± 28
27.3 (2)‡	77.5 (5)‡		

* Artificially spawned 4 days previously.
† Artificially spawned 3 days previously.
‡ Pooled plasma. Figure in parentheses = number of fish.

Corticoid values are shown in Table 3. Three spawning grilse taken at Coleman Hatchery exhibited a corticoid concentration of 29.6 μg/100 ml.

(4) *Spawning steelhead trout:* Plasma 17-OHCS levels were determined in 13 males and 14 females (Table 4). Six individual males had a mean value of 21.6 ± 5 μg/100 ml and values of 26.2 and 23.2 μg/100 ml on 2 and 5 pooled plasmas,

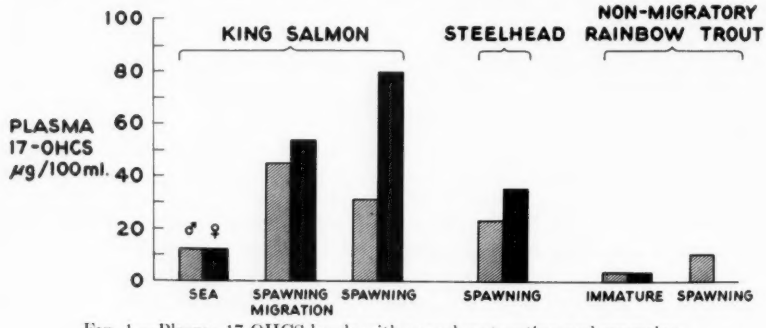

FIG. 1.—Plasma 17-OHCS levels with sexual maturation and spawning.

TABLE 4

BLOOD LEVELS OF 17-HYDROXYCORTICOIDS (μG/100 ML) IN SPAWNING STEELHEAD TROUT

Males	Females
26.4	30.6
19.8	31.1
16.0	32.1
26.4	17.1
16.0	55.1
25.2	23.4
	24.1
	23.2
	41.5
	68.3
	26.8
	36.2
Mean 21.6 ± 5	34.1 ± 15
26.2 (2)*	36.5 (2)*
23.2 (5)*	

* Pooled plasma. Figure in parentheses = number of fish.

respectively. Twelve females had a mean value of 34.1 ± 15 μg/100 ml and 36.5 μg/100 ml. on a plasma pool of 2 fish.

(5) *Sexually immature and mature nonmigratory rainbow trout:* Heparinized blood was obtained by severing the tail of the trout. Pool plasma from 25 im-

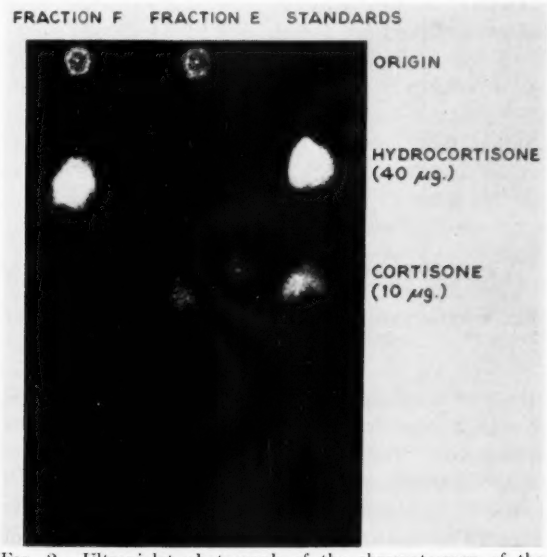

FIG. 2.—Ultraviolet photograph of the chromatogram of the salmon F and salmon E fractions.

mature males and females and a pool from 7 mature males showed 17-OHCS concentrations of 2.7 and 10.0 μg/100 ml, respectively.

Changes in the 17-OHCS levels with sexual maturation and spawning in both the males and females are summarized in Figure 1. Comparison is also shown

between the spawning king salmon, steelhead trout and nonmigratory rainbow trout.

Nature of the Adrenal Corticoids.—One hundred ml of pooled plasma from spawning female salmon containing 77.5 μg of 17-OHCS was extracted and subjected to paper chromatography in the Zaffaroni's chloroform-formamide system in an endeavor to identify the adrenal corticoids present. The areas corresponding to Compounds F, E (fractions F and E, respectively) and the area below E to the front (fraction B) were sectioned and eluted. Areas of fraction F and E from the first chromatogram were rerun on paper in Bush's toluene-methanol-water system. Figure 2 shows a photograph in ultraviolet light with fraction F and fraction E running in

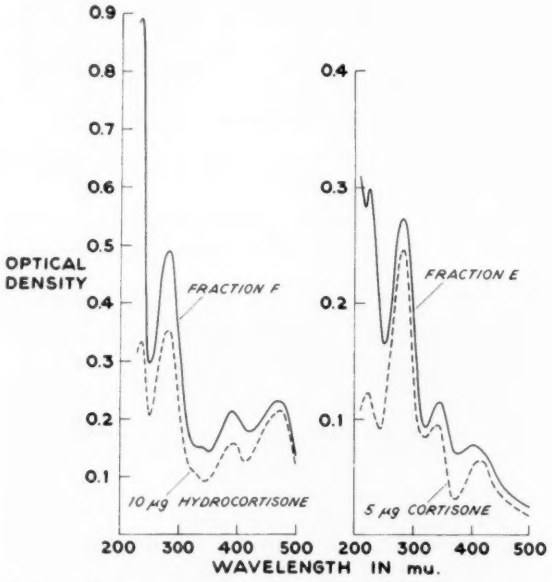

FIG. 3.—Absorption spectra of the sulfuric acid chromogens of salmon F and salmon E fractions.

parallel with the corresponding standards. It appears that the major steroid is hydrocortisone with a small amount of cortisone. Fraction B area was also run in the Bush system with corticosterone as the reference standard. No ultraviolet absorbing area corresponding to corticosterone was observed.

Areas of fraction F, E, and B were eluted from the paper and further analyzed. Fraction F had an Rf similar to the standard hydrocortisone. Aliquots of fraction F were checked by the "blue tetrazolium" reaction and the absorption spectra of the sulfuric acid chromogens. A positive "blue tetrazolium" reaction was obtained indicating an alpha ketol. Absorption spectra compared to that of hydrocortisone are shown in Figure 3.

Fraction E had an Rf similar to cortisone. Aliquots of fraction E were analyzed as described for fraction F. "Blue tetrazolium" reaction was positive, and the

absorption spectra was similar to that of standard cortisone as shown in Figure 3. Analysis of the fraction B area showed no detectable amounts of any of the $C_{21}O_4$ steroids.

Pooled male and female plasma from maturing salmon (Mill Creek), when chromatographed and analyzed, showed the same pattern of corticosteroids as the plasma from spawning female salmon. Similar analysis of spawning steelhead trout plasma gave analogous results.

TABLE 5

SUMMARY OF AVAILABLE DATA ON CONCENTRATION OF PLASMA 17-HYDROXYCORTICOIDS IN SEXUALLY MATURING AND SPAWNING FISHES

Reported by	Kind of Fish	Sex	During Sexual Maturation, μg/100 ml	At Spawning or Post-Spawning, μg/100 ml
Hatey,[14] Fontaine and Hatey[15]	Atlantic salmon	M	52.2	31.7
		F	45.3	30.6
Hane and Robertson	Pacific salmon (*O. tschawytscha*), Sacramento river	M	45.2	31.3
		F	53.4	78.7
Hane and Robertson	Pacific salmon (*O. tschawytscha*), Columbia river grilse	M	89.0	...
		F	...	...
Hane and Robertson	Rainbow trout, migratory (steelhead)	M	...	22.9
		F	...	35.2
Hane and Robertson	Rainbow trout, nonmigratory (hatchery)	M	...	10.0
		F	...	...
Bondy, Upton and Pickford[16]	Carp	M	...	24.4
		F	...	43.8

Discussion.—Very few studies of the adrenal corticosteroids in fishes have been reported. Phillips and Chester Jones[13] identified such steroids in the ray as corticosterone (8 μg/100 ml plasma), in the dogfish as hydrocortisone (2–3 μg/100 ml whole blood), in the cod as hydrocortisone (1 μg/100 ml whole blood), and in the lungfish as hydrocortisone (15 μg/100 ml plasma). No mention was made of the state of sexual development in these fish. The only determinations of 17-OHCS concentrations in salmon were made by Hatey[14] and Fontaine and Hatey.[15] These authors studied the plasma corticoids of young Atlantic salmon (*S. salar*) on their seaward migration and found 19.6 μg/100 ml in the parr and 85.5 μg/100 ml in the smolt. Their determinations on maturing and spawning salmon of the same species are given in Table 5, which summarizes the available data (including our own) on 17-OHCS levels in salmon, trout, and carp accompanying sexual development and spawning.

It will be noted that the corticoid concentrations (heart's blood plasma) of the two types of salmon, Atlantic and Pacific, during sexual maturation are essentially alike. However, at spawning the Atlantic salmon of both sexes showed a decided drop in 17-OHCS; whereas in the Pacific salmon, only the males showed this change. In contrast, the spawning female Pacific salmon exhibited a rise in corticoids to a much higher level. A similar, though less pronounced, difference in 17-OHCS concentration between male and female was found in the steelhead trout. Bondy, Upton, and Pickford[16] observed an analogous phenomenon in post-spawning carp. Histological examination of the adrenal cortical tissue of our spawning salmon and steelhead revealed no apparent difference in the degree of degeneration between males and females.

The progressive increase in the concentration of plasma 17-OHCS in the maturing

female Pacific salmon is not unlike that in the pregnant human female,[17, 18] in whom there is also hypertrophy of the adrenal glands.[19] While the mean levels of plasma 17-hydroxycorticosteroids in the third trimester of pregnancy found by the above authors (24 to 49 μg/100 ml) are lower than those of the sexually mature female salmon, the concentration rises at the time of labor and may reach 80 μg/100 ml[18] which is almost identical with our finding in the spawning salmon. The amounts of corticoids found in cases of Cushing's syndrome vary considerably, but in many such cases plasma levels are approximately the same as those in sexually maturing salmon, i.e., 40–50 μg/100 ml.

The increasing concentrations of 17-OHCS in the blood of the Pacific salmon as they progress from the sea to the spawning grounds parallels in general the inception and degree of adrenal hyperplasia described in an earlier study.[5] By the time the salmon was ready to spawn the amount of adrenal cortical tissue had increased many fold over that present in the immature fish in the sea. The hyperplasia appeared to be equally pronounced in males and females.

The results of this study do not reveal any unequivocal relationship between height of 17-OHCS concentration and survival after spawning among the salmonids, with the possible exception of the nonmigratory rainbow trout. These fish usually survive their first spawning with a corticoid concentration of only 10 μg /100 ml. Steelhead trout and Atlantic salmon both exhibit a considerable post-spawning mortality and have an elevated plasma corticoid content. Yet carp, which are known to spawn repeatedly, have been shown to possess an equally high 17-OHCS concentration.

The significance of the well-established marked increase in the plasma concentration of adrenal corticosteroids lies in their catabolic effects on the body organs and tissues. No information on this subject in fish is available. Forthcoming communications will deal with histological and physiological changes associated with these abnormally high concentrations of 17-hydroxycorticosteroids.

Summary—Plasma 17-hydroxycorticosteroids have been determined in king (Chinook) salmon at successive stages of sexual development from complete immaturity in the sea to full maturity on the spawning grounds. Plasma samples taken at two points on the spawning migration showed a marked rise in 17-OHCS concentration in both males and females of 4 to 5 times the normal value at sea of 11.8 μg /100 ml. At spawning the females showed a further rise to a mean of 79 μg /100 ml, while the concentration in the males fell to 31 μg /100 ml. Chromatographic studies of the plasma of sexually mature salmon revealed the presence of hydrocortisone and cortisone, predominantly the former. Absorption spectra of the sulfuric acid chromogens and Rf values gave further confirmation of the nature of these two corticoids. No other corticosteroids were detected. Concentrations of 17-OHCS in the plasma of spawning anadromous rainbow trout (steelhead) averaged 23 μg /100 ml for the males and 35 μg /100 ml for the females. Chromatographic analysis demonstrated the same corticoids as in the salmon. In sexually mature nonmigratory male rainbow trout (hatchery reared) a concentration of only 10.0 μg /100 ml was present.

The relationship of the plasma corticoids to the degree of adrenal hyperplasia has been discussed.

* This study was aided by a grant (#*G*-3324) from the National Science Foundation.

† We wish to express our sincere appreciation to the following members of the California Department of Fish and Game for their generous assistance in securing material for this study: Dr. Alex Calhoun, Mr. Leo Shapovalov, Mr. Richard Hallock, Mr. Phillip E. Pister, Mr. Ralph V. Beck, Mr. Eugene R. German, Mr. William Van Woert, Mr. John E. Riggs, Mr. T. James Ready, Mr. Carleton Rogers, and Mr. Norman Randolph. We also wish to thank Mr. John Pelnar, Coleman Station, U. S. Fish and Wildlife Service at Anderson, California, and Mr. Roger E. Burrows of the same service at the Salmon Cultural Laboratory, Entiat, Washington, for their valuable help in providing migrating and spawning salmon. For their generous assistance in securing blood specimens on various field trips, we are greatly obliged to Dr. Sydney F. Thomas, Dr. Marcus A. Krupp, and Dr. Cutting B. Favour of the Palo Alto Medical Research Foundation, Palo Alto, California. The authors are also indebted to Dr. P. H. Forsham, Director of the Metabolic Unit, and Dr. V. C. DiRaimondo of the University of California Medical Center, San Francisco, for their guidance and pertinent criticisms throughout the course of this investigation. The technical assistance of Mr. Shiro Horita is gratefully acknowledged.

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[12] Due to the rapidity with which the salmon were caught on the collecting trip to the Bolinos Bay region, the interval between capture and bleeding (followed by autopsy) became progressively longer. In spite of the fact that the fish were held in a large tank supplied with an abundant flow of fresh sea water many of them were listless and some lying on their sides by the time we were able to bleed them. The last fish, bled after 2 hours, was quite immobile. There was a progressive rise of the Porter-Silber chromogens with time after capture from 7.0 (1 fish), 13.2 (mean of 7 fish), 23.8 (mean of 9 fish), to 29.2 (1 fish) μg/100 ml at 2 hr. In the other experiments reported in this paper less than 10 min elapsed between catching and bleeding.

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Addendum: As this paper was going to press our attention was called to a report by Idler, Ronald, and Schmidt on the isolation of cortisone and cortisol from the blood plasma of Pacific salmon (*Oncorhynchus nerka*) which appeared in the March 5, 1959 *J. Am. Chem. Soc.* These workers found 17μg of hydrocortisone (cortisol) and 37 μg of cortisone per 100 ml plasma in salmon just before arrival at the spawning ground. This ratio of the two corticoids is the reverse of that present in the blood of the king salmon *O. tschawytscha*.

THYROID RADIATION DOSES FROM FALLOUT

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Communicated by A. H. Sturtevant, April 20, 1959

Absorbed doses of radiation from fallout, when averaged over yearly periods and over large numbers of individuals, have usually been assumed to be well below the corresponding doses from natural background sources. Although this assumption may be valid for most body organs, it is unlikely to have been valid for the thyroid glands of the average infant and child in the U.S. The purpose of this note is to point out that for the last few years such thyroids probably have received an annual radiation dose (chiefly, from the beta-rays of radioiodine) that is approximately equal to the annual dose which they receive from natural background radiations.

The radioiodines in fallout (chiefly, iodine-131, with a half-life of 8.05 days) pose a special hazard to infants and children. This arises in part because radioiodine tends to concentrate in fresh cow's milk[1, 2]—a major item in the diet of young people. Upon ingesting milk contaminated with radioiodine, human beings are expected to concentrate the isotope in their thyroids. Moreover, for the same amount of iodine-131 orally ingested, the average infant receives, according to estimates by Halnan and Pochin,[3] about 18 times the thyroid dose that the average adult receives.[4] This 18-fold increase results from the smaller size and possibly somewhat higher iodine uptake[3, 5] of the infant thyroid, and from the almost complete absorption of the iodine-131 beta-rays even within the small infant gland. Finally, there is evidence that the thyroid gland of infants and children is especially sensitive to radiation-induced carcinogenesis.[6]

Estimates of fallout doses to thyroids of infants and young children can be made by considering measurements of radioiodine activity levels in milk. Recently, Campbell *et al.*[7] have published such data for milk samples taken once a month at a sampling station in each of five metropolitan milksheds in the U.S. The radioiodine activities of such samples fluctuated widely with time and with locality. For a one-year period ending June, 1958, the over-all average for the five milksheds was 150 $\mu\mu$c per liter. From a more recent report,[8] it may be calculated that for a 16-month period ending September, 1958, the corresponding average had declined to 122 $\mu\mu$c per liter. In order to have an estimate for this 16-month period that takes into account the higher population density in the eastern U.S. and that avoids giving too much weight to extreme fluctuations from less densely populated areas (namely, from a low of 41 $\mu\mu$c per liter for a milkshed in the far West to a high of 219 $\mu\mu$c per liter for one in the Middle West), the U.S. average for this period is taken as that of a milkshed in the East (New York City). The latter average was 64 $\mu\mu$c per liter, or roughly one-half the average for the five milksheds combined.

Continued ingestion of milk contaminated at an average iodine-131 level, L (in $\mu\mu$c per liter), is expected to give at equilibrium a thyroid dose rate R (in rads per year), which can be expressed as follows:

$$R = \frac{0.039 \cdot L \cdot C \cdot U}{M}$$

where C is the average daily consumption of fresh milk in liters; U is the fraction of ingested radioiodine taken up by the thyroid; and M is the thyroid mass in grams.[9] Rough estimates of the values of these parameters for average infants and children in the U.S. are shown in Table 1.

Values of L are expected to be lower than the aforementioned observed iodine-131 levels in milk by a decay factor of about 0.9; that is, the elapsed time between collection of the milk sample at the sampling station (the arbitrary reference point for calculating radioiodine activities[7]) and human ingestion is presumably one to two days. This gives a value of L of 60 $\mu\mu$c per liter for the average U.S. value and a

TABLE 1

EVALUATION OF AVERAGE THYROID DOSE RATES (R) BY AGE OF INDIVIDUAL FOR A 16-MONTH PERIOD COMMENCING JUNE, 1957. THE ESTIMATED RANGE OF THE AVERAGE VALUES IS SHOWN IN PARENTHESES

Age of Individual	L = Average Milk Iodine-131 Levels, $\mu\mu$c per liter	C = Average Rate of Fresh Milk Consumption, liters per day	U = Fraction of Radioiodine Taken Up by Thyroid	M = Average Thyroid Mass, gm	R = Thyroidal Dose Rate, rads per year, $= \frac{0.039 \cdot L \cdot C \cdot U}{M}$
0–1	60	0.3	0.3	1.9†	0.11
	(35 to 200)*	(0.2 to 0.6)	(0.2 to 0.5)		(0.03 to 1.2)‡
1–2	60	0.7	0.3	2.5	0.20
	(35 to 200)	(0.5 to 0.9)	(0.2 to 0.5)		(0.05 to 1.4)
2–3	60	0.7	0.3	3.4	0.14
	(35 to 200)	(0.5 to 0.9)	(0.2 to 0.5)		(0.04 to 1.0)
3–5	60	0.7	0.3	5.1	0.10
	(35 to 200)	(0.5 to 0.9)	(0.2 to 0.5)		(0.03 to 0.7)
5–10	60	0.7	0.3	8.6	0.06
	(35 to 200)	(0.5 to 0.9)	(0.2 to 0.5)		(0.02 to 0.4)

* Range of average values for five milksheds, corrected to time of milk ingestion.
† Range of values not known.
‡ Range calculated by compounding ranges shown in preceding columns.

range of 35 to 200 $\mu\mu$c per liter for the five milksheds. Estimates of the values of C are taken as 0.3 liter per day for the first year of life, and 0.7 liter per day thereafter up to age ten.[10] These values are subject to considerable uncertainty and are based on the consideration that the range of values of C is expected to be between one pint and one quart per day, except during the first year of life when fresh milk consumption may well be lower owing to the widespread use of evaporated milk in infant feeding programs.[11] On the basis of measurements on normal infants and children by Oliner *et al.*,[5] U is taken to be 0.3 (however, Halnan and Pochin[3] estimate a value of 0.45 and some earlier studies summarized by Oliner *et al.*[5] gave values below 0.3). Estimates of the values of M are based on measurements compiled by Boyd.[12]

From Table 1, it can be seen that the calculated values of R for average infants and children in the U.S. during the 16-month period commencing June, 1957, depend upon age and vary from 0.06 to 0.2 rad per year. It should be noted that actual thyroid dose rates during this period may have been slightly higher than the calculated values of R, since there must also have been a dose contribution from the shorter-lived radioiodines in fallout[13] and from inhaled radioiodine.[14] For each age group the estimated range in values of R (shown in parentheses in Table 1) is considerable, depending chiefly upon observed geographical variation in milk radioiodine levels and upon uncertainties as to the true average values for rate of milk consumption and radioiodine uptake. On an individual basis the range in

values of R would be somewhat greater than that shown in Table 1, since there are expected to be greater individual variations in the values of L, C, U, and M. Since the monthly variation in milk radioiodine levels was observed[7] to range from lows of 0 to highs (in two of the milksheds) of over 900 $\mu\mu$c per liter, thyroidal dose rates to infants and children consuming such milk probably fluctuated widely on a short-term basis. For example, on a weekly basis, such dose rates would be expected to have ranged from 0 to about 0.1 rad per week during the 16-month period under consideration. Moreover, dose rates to different parts of the thyroid gland may fluctuate considerably on a short-term basis, owing to the possibility of non-uniform distribution of the radioiodine, as discussed by Oddie.[15]

Prior to June, 1957, data on milk levels of radioiodine activity are not available. However, cattle and human (largely adult) thyroid radioactivity measurements have been related to nuclear testing patterns for part of the year 1954 and for the years 1955 and 1956.[2, 16] In addition, the number and timing of such tests during 1957–1958 have been reported in relation to observed iodine-131 levels in milk.[7] On the basis of such information, it appears that for several years prior to 1957 annual thyroid doses to the average infant and child in the U.S. may have been similar to those calculated above for the 16-month period commencing June, 1957.

Natural background radiations are believed to contribute an absorbed dose to soft tissues of about 0.1 rad per year.[17] From the above account it appears that for the last few years the radioiodines in fallout have contributed an annual dose to the thyroids of average infants and children in the U.S. that is similar to, or in some circumstances greater than, the annual dose to such organs from natural background radiations.[18]

Summary.—On the basis of published data on iodine-131 levels in cow's milk during a 16-month period ending September, 1958, the thyroid glands of average infants and children in the U.S. are estimated to have experienced over the last few years annual doses of about 0.1 rad to 0.2 rad from the radioiodine in fallout. These doses are roughly one to two times the annual dose to such organs from natural background radiation. For a number of reasons, individual thyroid dose rates from fallout are expected to show wide deviations from the average rate.

[1] See review of cattle radioiodine studies by C. L. Comar, and R. H. Wasserman, in *Progress in Nuclear Energy, Ser. VI* (London: Pergamon Press, 1956), 184–196.

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[4] It is this fact which seems to have been overlooked in evaluating fallout doses of radioiodine. However, the importance of this fact was recognized in the case of the Windscale reactor accident in England; see, for example, Burch, P. R. J., *Nature*, **183**, 515 (1959).

[5] Oliner, L., R. M. Kohlenbrener, T. Fields, and R. H. Kunstadter, *J. Clin. Endocrinol. and Metab.*, **17**, 61 (1957).

[6] This evidence is based chiefly on follow-up studies by C. L. Simpson and L. H. Hempelmann, *Cancer*, **10**, 42 (1957) of infants irradiated about the chest with doses of several hundred r of X-rays; and follow-up studies by Sheline, G. E., S. Lindsay, and H. G. Bell, *J. Clin. Endocrin. and Metab.*, **19**, 127 (1959) of children and adults receiving radioiodine therapy for hyperthyroidism. See also reviews by Duffy, B. J., Jr., *J. Clin. Endocrin. and Metab.*, **17**, 1383 (1957) and Rooney, D. R., and R. W. Powell, *J. Am. Med Assoc.*, **169**, (1959). There is some evidence from these

sources that the infant thyroid, in spite of its much smaller size, is more sensitive to radiation-induced carcinogenesis than the adult thyroid.

[7] Campbell, J. E., G. K. Murphy, A. S. Goldin, H. B. Robinson, C. P. Straub, F. J. Weber and K. H. Lewis, *Am. J. Public Health*, **49**, 225 (1959).

[8] "Strontium Program—Quarterly Summary Report," *Health and Safety Laboratory Document HASL-55*, U.S. Atomic Energy Commission, New York Operations Office (1959).

[9] The evaluation of the constant in this equation is based on physical and biological data for iodine-131, which have been summarized, for example, in "Recommendations of the International Commission on Radiological Protection," *Brit. J. Radiol.*, Supplement No. 6 (1952). It is assumed that the effective half-life of iodine-131 is 7.7 days and that the effective beta energy is 0.19 Mev.

[10] A recent survey of 312 children under the age of six in suburban Long Island by H. H. Neumann, *Arch. Pediat.*, **74**, 456(1957), gave a mean value of 840 ml of fluid cow's milk consumed per day with the lower third of the group averaging 647 cc per day and the upper third averaging 1,005 cc per day.

[11] Since evaporated milk will tend to be several months old before it is consumed, its iodine-131 activity should be virtually nil.

[12] Boyd, E., in *Handbook of Biological Data*, ed. by W. S. Specter (Philadelphia: W. B. Saunders Co., 1956).

[13] Dunning, G. M., *Nucleonics*, **14**, No. 2, 38 (1956).

[14] See, for example, R. L. Gunther and H. B. Jones, *U.S. Atomic Energy Commission Document UCRL-2689 and addendum* (1954).

[15] Oddie, T. H., *Brit. J. Radiol.*, **24**, 333 (1951).

[16] Middlesworth, L. Van, *Nucleonics*, **12**, No. 9, 56 (1954) and *Science*, **123**, 982 (1956); Comar, C. L., B. F. Trum, U. S. G. Kuhn III, R. H. Wasserman, M. M. Nold, and J. C. Schooley, *Science*, **126**, 16 (1957).

[17] Spiers, F. W., *Brit. J. Radiol.*, **29**, 409 (1956).

[18] It should be noted that with the cessation of nuclear weapons testing in November, 1958, human thyroid levels of radioiodine should decline exponentially and by January, 1959, there should no longer have been any appreciable contamination of milk with radioiodine from past weapons tests. The level of radioiodine in cow's milk may well be the most important index of short-term environmental contamination with fission products whether from weapons tests or other sources [see discussion in *Safety Aspects of Nuclear Reactors*, ed. C. R. McCullough (New York: D. Van Nostrand Co., 1957), 28].

THE ELIMINATION OF DNA FROM SOMA CELLS

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Read before the Academy, April 29, 1959

The current widespread interest in the role of DNA in protein synthesis, on the parts of cytologists and biochemists alike, brings into question anew a very old enigma of cytology, which is, the reason for the elimination of parts of or whole chromosomes from somatic cells or nuclei of embryos so that in some animals the chromosome complex of germ cells is markedly different from that of the soma cell of the same individual. In the fungus-feeding fly, *Oligarces paradoxus*, for example, germ cells show 66 chromosomes while soma cells contain only 10 such elements (Reitberger[1]).

The loss of parts of chromosomes from soma cells was first observed by Boveri[2] in the cleaving eggs of *Ascaris megalocephala*, and sporadically through the years other examples of such aberrant behavior have been recorded. The elimination

of whole chromosomes from soma cells occurs regularly in species of Diptera belonging to the families Cecidomyidae, Chironomidae, and Sciaridae.

The very intriguing question, which has aroused much speculation since Boveri's original observations is: What useful purpose is served by the elimination of DNA from soma cells during early embryonic development? The fact that species in which this regularly occurs are spread all over the earth suggests that this anamolous behavior must have an adaptive value else it would not have become established.

Many different suggestions have been made to account for DNA elimination from soma cells but none has been free from objection nor subject to experimental verification (for a recent review see Goldschmidt[3]). It is clear, however, that the persisting somatic chromosomes (hereafter designated S-chromosomes) carry all the genetic factors needed for the normal development of adult males and females, from which it may be inferred that the eliminated chromosomes (E-chromosomes) have no immediate genetic function.

Today, in the light of a better understanding of the functions of DNA and especially the new discoveries in nuclear chemistry, it becomes possible to correlate seemingly unrelated facts, and there emerges a plausible and very convincing explanation which not only allows us to understand the meaning of DNA elimination but sheds some light, perhaps, on the role of heterochromatin in the economy of cells. The writer believes that the evidence to be reviewed below justifies the conclusion that the excess DNA carried in germ cells of certain flies and in at least one genus of plants, plays an essential role in building up, in the cytoplasm of oocytes or the seed, part of the proteins and nucleic acids (both DNA and RNA) required for the development of embryos. In short, it is an ovarian adaptation to meet a specific need. The subsequent elimination of an excess of DNA from soma cells is highly advantageous to the organism because it frees the soma cells from the necessity of synthesizing anew, or reassembling, nucleotides which are not required for somatic development. In this way, the supply of nucleic acid precursors previously stored in the cytoplasm of the female gamete is conserved and the more rapid development of the embryo is made possible.

Biologists have always understood, of course, that in oviparous animals the mature egg must carry within itself all the food reserves needed for the development of the embryo to the point when it can get additional food from its environment. And before the turn of the present century cytologists had recognized and described two different cellular mechanisms by which essential foodstuffs are synthesized and stored in the egg's cytoplasm. At one extreme the synthesis of proteins, nucleic acids and other materials is carried out by specialized nutritive cells—nurse cells—and subsequently this material is transferred to the egg's cytoplasm in several ways. In such cases the egg nucleus remains small until late in oocyte development and appears to play a minor role in syntheses. At the other extreme, in the absence of typical nurse-cells, the egg's nucleus itself becomes enormously enlarged to a germinal vesicle and is extremely active in synthesizing proteins and nucleic acids. In some animals the oocytes show both nurse-cell and germinal vesicles. Of especial interest are species of mosquitoes in which the adult female must feed upon blood before oocyte development is complete, for it has been shown that a mixture of essential amino acids may be substituted for a blood meal. Obviously, the problem of getting enough raw materials from which to synthesize egg cyto-

plasmic constituents, especially proteins, is a critical one for all organisms. Examples of these two synthetic mechanisms will be helpful in considering the main questions before us.

In the ovary of the fruit fly, *Drosophila melanogaster*, an early step in oocyte development is the formation of the egg chamber containing 15 nurse-cells and one oocyte. The nurse-cells, which are modified oogonia, now undergo a series of endomitotic division cycles during each of which the chromosome number is doubled so that the largest nurse-cells may carry from 512 to 1024 haploid sets of chromosomes (Painter and Reindrop[4]). There is a corresponding increase in cytoplasmic volume after each endomitotic division cycle. Nucleoli are extremely numerous and voluminous in nurse-cells and since, as Vincent[5] has shown, nucleoli are composed mainly of proteins in a highly dehydrated state, it is obvious that nurse-cell nuclei synthesize large amounts of nuclear protein. Eventually, the contents of all nurse-cells are incorporated into the cytoplasm of the oocyte, sometimes by a breakdown of the cell wall with direct absorption but more usually by indirect absorption. As a result of the absorption process the cytoplasm of the mature egg contains a large amount of protein synthesized in the nuclei of nurse-cells as well as in the cytoplasm of the oocyte and in addition the degradation products (probably depolymerized) of many thousands of chromosomes.

In the ovary of the frog or toad the story is quite different as to details, but in the end the same situation obtains as in the insect egg. Germ cells pass through the early stages of meiosis up to the diplotene stage without a very great increase in nuclear or cytoplasmic volume. But from this point on, for a period of months there is a consistent increase in nuclear and cytoplasmic volume until the nucleus reaches a diameter of about 150 microns and the whole egg is a millimeter or more in size. During this long period very significant changes occur within the nucleus. In the leptotene stage Painter and Taylor[6] reported that in addition to the thread-like chromosomes, and quite separate from them, many hundreds of Feulgen-positive granules lie just within the nuclear wall forming a sort of cap of DNA particles on the side of the nucleus opposite that toward which the zygotene threads are oriented. In early pachytene small nucleoli, rich in RNA, arise in direct contact with one or more of the independent DNA granules. Such nucleoli with the DNA and RNA components persist for a time and then begin to disappear. First, the DNA granule fades, then the RNA and finally the proteins of the nucleolus seem to melt away. Concomitant with nucleolar disappearance, a gradient of RNA builds up in the cytoplasm which is densest nearest the nuclear wall. After the DNA granules visible in the leptotene stage are used up, and all through the months of the germinal vesicle growth, new crops of nucleoli appear. These are rich in RNA and apparently arise initially in close association with heterochromatic regions of the diplotene chromosomes, and often (always?) a DNA granule is attached to the small nucleolus. We seem to be dealing with a dynamic process during which the chromosomes shed nucleolar material and this passes to the nuclear wall and disappears.

From the above sequence of events Painter and Taylor concluded that, in ways not then understood, the nucleolar material (consisting mainly of RNA and concentrated proteins) passed through the nuclear wall into the cytoplasm of the oocyte. Recently, very striking support for this inference has come from two different

sources. Hoff-Jorgensen,[7] using a bioassay method, has shown that the cytoplasm of the frog's egg contains over 4,500 times as many nucleotides (measured as thymidine) as the haploid nucleus of a sperm, from which it is evident that the DNA granules, attached to nucleoli, pass out into the cytoplasm. Recently, some excellent electron photographs have been published of the germinal vesicle of the frog's egg, both by Kemp[8] and Wischnitzer,[9] and these clearly show that the disappearance of nucleoli, as seen by the light microscope, is due to a shredding of the nucleolar material into granules about 150 A in diameter. That such granules pass through the extremely abundant pores in the wall of the germinal vesicle is strongly supported by Kemp's figures.

From the evidence presented it is clear that in the growth of oocytes large quantities of DNA are required for the synthesis of nuclear proteins, whether the actual site of synthesis be in small, separate factories (nurse-cells) or in one large center, the germinal vesicle. Viewed in this light it seems extremely probable that in those families of Diptera, which carry high numbers of chromosomes in their germ cells, as compared to the soma complexes of the same individual, the extra E-chromosomes are used to synthesize nuclear proteins and thus correspond functionally to the DNA granules of the frog's egg. The entire behavior of the E-chromosomes both in meiosis and in early cleavage is consistent with this interpretation.

In the Cecidomyidae, the family of gall-flies which has been most extensively studied cytologically by White,[10] the behavior of the E-chromosomes during meiosis is quite different in males and in females. During sperm formation all the E-chromosomes (with exception of one transitional species) are eliminated during the first division so that mature sperm carry only a haploid number of S-chromosomes. During the meiosis of females both a haploid number of S-chromosomes and representatives of all the E-chromosomes are found in the female gamete. After fertilization the zygote carries a diploid number of S-chromosomes and representatives of all the E-chromosomes derived from the mother. During early cleavage stages, usually the third, the E-chromosomes are eliminated from the spindles of potential somatic nuclei but are retained in the most posterior nucleus, which becomes the germ cell.

According to White adult females do not require feeding prior to laying mature eggs. Incidentally, in the Cecidomyidae, to judge from published figures, both nurse cells and a germinal vesicle function.

In the dog-roses of Europe a somewhat similar situation exists as in the gall-flies. In *Rosa canina* and related species, there are 35 chromosomes in both male and female germ cells, consisting of 7 pairs of homologous chromosomes (corresponding to S-chromosomes) and 21 univalent elements (E-chromosomes). During the meiosis of male germ cells all the univalent E-chromosomes are lost so that functional pollen carries only a haploid set of S-chromosomes. In female germ cells the mature gamete carries a haploid number of S-homologues and a full complement of univalent E-chromosomes. The later development of the so-called embryo-sac is quite complicated but one cell functions as a female gamete and is fertilized and the rest of the cells, including triploid nuclei become a part of a cytological mechanism for synthesizing the food material of the seed. Thus in plants the fundamental requirement of providing food for the development of the

embryo is delayed until after fertilization but, as in animals, much DNA is required for synthetic processes.

It goes without saying that the elimination of parts of chromosomes in early cleavage from potential soma cells may be explained in the same way as the elimination of whole chromosomes.

The recent work of Allfrey and Mirsky[11] greatly contributes to the understanding of reserve food mechanisms found in the oocytes of animals. These authors have shown three important facts: (1) that DNA is essential for the synthesis of proteins in the nucleus; (2) that as DNA is removed from thymocyte nuclei, by the use of DNase, in measure, the ability of nuclei to synthesize protein falls off; (3) that in a nucleus depleted of its DNA, the ability to synthesize proteins can be partially restored by adding to the medium of such nuclei a polyanion which is not related structurally to nucleic acids. These authors point out that the role of DNA in protein synthesis may be due, in part, to the configuration of the DNA molecule.

Applying the above chemical information to the nurse-cell and germinal vesicle mechanisms, it may be said that the ability of a nucleus to synthesize proteins depends on the amount of DNA it contains, or on the amount of a special kind of DNA. In nurse-cells the repeated reduplication of chromosome sets by endomitosis greatly increases the "nuclear horsepower" of each nurse-cell so that the ability to synthesize proteins of the largest of such cells in the ovary of the fruit fly may be from 256 to 512 times greater than that of a diploid nucleus. Similarly, the initial presence of extra chromosomal granules in the young germinal vesicle of the frog greatly increases the protein synthesizing power of this cell component.

It appears quite clear, from the evidence reviewed above, that in the growth of oocytes, much more DNA is needed for the synthesis of nuclear protein than can be supplied by the diploid or, after synapsis, the tetraploid nucleus. One way of meeting this need is for the germ cells to carry an excess of DNA—in the form of the E-chromosomes—but there are serious disadvantages of having such extra DNA in all cells of the organism where it is not needed. First of all, in male gametes, whether sperm or pollen, lightness of weight or small size would presumably have an adaptive value. In soma cells, were the E-chromosomes retained in all nuclei during embryonic development, the replication of DNA structures not needed for development would impose a heavy drain on the supply of nucleotides or other nucleic acid precursors stored earlier in the cytoplasm of the oocyte. From this the advantages of eliminating unnecessary material are very clear.

The question may now be raised, What becomes of the discarded chromatin, i.e., mainly DNA? The rapid disappearance of whole or parts of chromosomes from cytoplasm is undoubtedly due to the presence of DNase in the cytoplasm, but it appears to the writer that nucleic acid precursors are much too valuable a material to be discarded. In this relation the insect egg is admirably adapted for the conservation of discarded DNA because cell walls are not formed in the embryo until some 2000 nuclei are present, so all such nuclei would have free access to deoxyribotides or -sides. This would greatly hasten development of the soma cells, as I pointed out some years ago (Painter[12]).

The elimination of DNA from soma cells raises many questions, chief of which is the origin of the extra DNA found in germ cells. Since in *Rosa canina* the 35 chromosomes present in germ cells is known to be a pentaploid complex ($n = 7$

chromosomes) several writers have assumed that in animals the high chromosome number of germ cells is a derivative of an originally polyploid condition. At the moment this appears as the most likely explanation.

A second, and related question, is raised by the fact that the chromatin thrown out of the spindle, during the chromatin diminution in *Ascaris*, has come to be regarded as being made up of nongene bearing chromatin, i.e., is heterochromatin. Are the 55 extra chromosomes in the germ cells of *Oligarces*, for example, made up wholly of heterochromatin? No answer can be given to this question but since the original findings of Muller and Painter[13] in 1932, it has been evident that not all the DNA along the chromosomes is gene-bearing and now the striking experiments of Mirsky and Allfrey suggest a way in which DNA may lack the specificity of the gene-bearing areas and still contribute to the synthesis of nuclear protein.

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